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Measurement of gas exchange rates in plant tissue culture vessels

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Abstract

The aerial microenvironment in culture vessels has a significant effect on the growth and development of plantlets *in vitro*. Since the gas exchange between outside air and inner air can influence the microenvironment of culture vessel, it is necessary to measure the air exchange rate for various vessels. In this study, water vapor was used as the tracer gas, and the change of absolute humidity inside the vessel was calculated continuously by the measured values of a relative humidity sensing element. The outside environment was maintained at constant humidity level by a saturated salt solution. The RH data were transformed into absolute humidity and the specific humidity ratio. The air exchange rates of several tissue culture vessels were then calculated. The exchange rate was between 0.0145 h^{-1} to 0.0376 h^{-1}. This technique provides an inexpensive, rapid and simple way to determine the air exchange rate of a culture vessel within a short period. The effects of the air current velocities on the exchange rates of vessels were also determined.

Introduction

Plantlets *in vitro* are cultured in small culture vessels. The water and nutrition are supplied by the medium. The special microenvironment within this system are high relative humidity, near stable temperature, low CO_2 concentration in light and high CO_2 concentration in dark, and high C_2H_4 concentrations (Kozai et al., 1996). CO_2 concentrations may drop to become a limiting factor for plant growth in the light period and high C_2H_4 concentrations inhibit growth of plantlets and induce senescense (Buddendorf-Joosten and Woltering, 1994). High relative humidity reduces the transpiration and induces stomatal malfunction (Ghasshghaie et al., 1992). The growth conditions of plantlets are significantly affected by the inside microenvironment of culture vessels. However, the microenvironment of culture vessels is not easily adjusted.

Explants are transplanted into small and semi-closed culture vessels, and growing conditions require a low density (or even absence) of microorganisms. Thus it is impractical to circulate the inside air by ventilation equipment as in greenhouse production. A way to modify the microenvironment of vessels is to exchange the gas between inside and outside environments.

The number of gas exchanges of the greenhouse per minute is a widely used measurement in greenhouse engineering. Because the small magnitude of gas exchange of a vessel, the number of exchanges of the vessel per hour is adopted, and is defined as the hourly volumetric ventilation rate of the vessel divided by the air volume of the vessel (Kozai et al., 1986). The factors affecting this parameter include the shape and physical properties of cover and vessel, tightness of the lid and air velocity and direction outside the vessel, etc.

There have been only a few papers reporting the measurements of gas exchange rates of tissue culture vessels. Kozai et al. (1986) first proposed a method to determine the effects of the stoppers and vessels in gas exchange rates. For this, they used CO_2 as a tracer gas, with high concentrations of CO_2 inserted into the vessel. The gas concentration inside the vessel at certain time intervals was measured with a

gas chromatograph. Two sets of data were selected to estimate the number of gas exchange rates by a previously developed equation.

Jeong et al. (1993) developed this technique in more detail. They first replaced the inside air of vessel with air having a CO_2 concentration higher than 10 ml l^{-1}. This vessel was placed in the tissue culture room. Air samples were taken from the vessel at two different times, their CO_2 concentrations were measured using gas chromatography, and the gas exchange rate was then calculated. Ibaraki et al. (1992) noted that using this gas sampling technique, the air microenvironment of the culture vessel could be disturbed, therefore they proposed another technique of filling higher CO_2 concentration gas into the vessel, and then weighing the culture vessel at regular intervals with a precise electric balance. The change of CO_2 concentration was then calculated by the change of weight. However, this method was limited by the accuracy of the electric balance. Both the gas chromatography and electric balance technique are relatively expensive and these previous studies determined the gas exchange rates of culture vessels by using only a few data.

The air exchange between the inside microenvironment and outside environment is due to the difference of gas concentration. Many kinds of gases comprise the air in culture vessels, such as CO_2, O_2, C_2H_4, N_2 and water vapor, and all of these can be adopted as a tracer gas (Fujiwara and Kozai, 1995). In this study, the water vapor was adopted to measure gas exchange as a rapid and easy technique using an inexpensive and precise relative-humidity sensor.

Theoretical description of gas exchange rate

The driving force to enhance the gas exchange between inside and outside environments is derived from the different concentrations of gases (Geankoplis, 1993). To develop a model of gas exchange rate, some conditions are assumed as follows (Fujiwara and Kozai, 1995):

1. Air temperature and pressure inside a vessel are the same as those outside of the vessel.
2. The gas is uniformly distributed in the vessel.
3. The gas is not absorbed or released in the wall of vessel.

The diffusion process of a gas in a vessel between inside and outside environment can be described as:

$$dc_1 = K_c[C_{out} - C(t)\mathrm{d}t \quad (1)$$

where, C_{out} is the gas concentration outside the vessel; $C(t)$ is the gas concentration inside the vessel at time t; and K_c is the gas exchange rate of vessel.

In this study, neither medium nor plantlet was contained in the vessel, so the gas exchange rates of plantlet and medium in the vessel is not a factor. At the initial state (t=0), the gas concentration in the vessel is C_i. C_{out} is maintained nearly constant. Equation (1) can be integrated and rearranged to give the equation:

$$C(t) = C_{out} + C_i - C_{out}\,\mathrm{Exp}[-K_c \times t] \quad (2)$$

The gas concentration ratio, CR(t) is defined as:

$$\mathrm{CR}(t) = \frac{C_{out} - C(t)}{C_{out} - C_i} \quad (3)$$

From Eqs. (2) and (3), and transformed CR(t) into natural logarithm form:

$$\ln(\mathrm{CR}(t)) = -K_c \times t \quad (4)$$

The K_c value of equation (4) can be calculated directly from two sets of data,

$$K_c = \frac{-1}{T}\ln\frac{C_1 - C_{out}}{C_2 - C_{out}} \quad (5)$$

where C_1 and C_2 are the CO_2 concentration at times 0 and t, respectively; and T is the time period between 0 and t. This equation was adopted by Kozai et al. (1986) and Jeong et al. (1994) to calculate the K_c values.

However, there are some problems about the K_c value calculated from Equation (5).

1. The plots of relationship between the gas concentration in the vessel and time period is an exponential curve, although Equation (5) uses only two data points of this continuous curve. The choice of two data points was in arbitrary. The measurement error of this data was not considered.
2. The outside gas concentration is not maintained as a constant, since it is always changed by the outside environment.
3. A gas chromatograph is required to ensure the accuracy of measured values of CO_2 concentration. However, this equipment is so expensive that it was not easy to be used in many laboratories.

In order to develop a convenient technique to measure the gas exchange rate of all kinds of plant tissue culture vessels, water vapor was selected to replace CO_2 as the tracer gas. The gas concentration for water vapor is absolute humidity (H, kg H_2O/kg dry air), and it can be easily calculated from relative humidity by the following equations (Albright, 1990):

1. Calculate the saturated vapor pressure , P_{ws} (kPa), at temperature Tk,

$$P_{ws} = \text{Exp}[-5800/Tk + 1.3915 - 0.04864Tk + 4.1765 \times 10^{-5}Tk^2 - 1.445 \times 10^{-8}Tk^3 + 6.546\ln(Tk)] \quad (6)$$

2. The vapor pressure (P_w) of air:

$$P_w = \text{RH} \times P_{ws} \quad (7)$$

3.

$$H = \frac{0.622 \times P_w}{P_{atm} - P_w} \quad (8)$$

where Patm is the atmospheric pressure, 101.325 kPa at sea level.

By the absolute humidity value, defined HR(t) as Equation (3), and proposed a new equation:

$$\text{HR}(t) = \frac{H_{out} - H(t)}{H_{out} - H_i} = \text{Exp}(-Kt) \quad (9)$$

Then,

$$\ln[\text{HR}(t)] = -Kt \quad (10)$$

Equation (10) is a type of linear equation without a constant term. Many sets of data pairs $(H(t), t)$, can be collected by a data logger. The K values of Equation (10) were then calculated by linear regression analysis technique with zero intercept (Weisberg, 1986), $K = -C$ and

$$C = \frac{\sum y_i t_i}{\sum ti^2} \quad (11)$$

In other words, the K value can be evaluated with all collected pair data by using regression analysis technique. Thus the deviations of K due to random error could be reduced.

Materials and methods

Culture vessels

Several types of vessel were selected to determine their gas exchange rates. These vessels are: triangular glass flask (CF1, I-Shin Co., Taiwan), Japanese irregular box (IW-1, Iwan, Japan), French box (PSCA-2, PSCA Co., French), GA-7 box (Magenta Co., USA), and Dutch box (LA-RH, LAB Associates, The Netherlands). A domestic box-type vessel (DCB-1, DCB Co., Taiwan) with four different treatments was also selected to observe the influence of treatments on the exchange rate. The model of gas permeable tape used in DCB-1 box was BRL. 10676-013, Gibco Co., USA.

Relative humidity sensors

Two types of relative humidity sensors are used in this study. The first type is a THT-B7 high polymer (macro molecule) sensor (Shinyei Co., Japan) which operates on the changing electric resistance of a sensing element according to relative humidity used to measure the inside relative humidity of small vessels. This sensing element is small (4.5×9.0×2 mm). Sensing signals are then transmitted to analog signals (Voltage) and recorded by a data logger. The accuracy of this sensor was improved within ±0.7% after calibrated.

The second type of relative humidity sensor is HOBO Hygro-logger (HOBO Company, USA). This device can simultaneously sense and record the humidity data. The size of this device is 35×35×15 mm, and its sensing element uses a high macro molecule material. The accuracy of this sensor was improved within ±1.0% after calibration.

The two types of sensor were made of non-hygroscope materials, so these sensors did not affect the humidity microenvironment of vessel.

Calibration of humidity sensors

The humidity sensors were calibrated by thirteen saturated salt solutions.

Experimental procedures

Two procedures were executed according to the open size of vessels.

1. Small open area (Figure 1)

The opening of the triangular flask was too small to install the HOBO Hygro-logger, so the THT-B7 sensing element was installed inside the vessel. The stopper of the flask was then cut, the connected line was inserted into the stopper, and the cutting position was sealed with Silicon. The procedures for the determination of gas exchange rate are as follows:

A. Measure the volume of flask, air temperature, and relative humidity.
B. Calculate the absolute humidity of the ambient air. Use the volume of flask and the difference between saturated absolute humidity and absolute humidity of ambient air, the added water required for the vessel to reach saturated condition was determined.
C. Add a predetermined quantity of water into vessel and rapidly seal the vessel with rubber stopper.

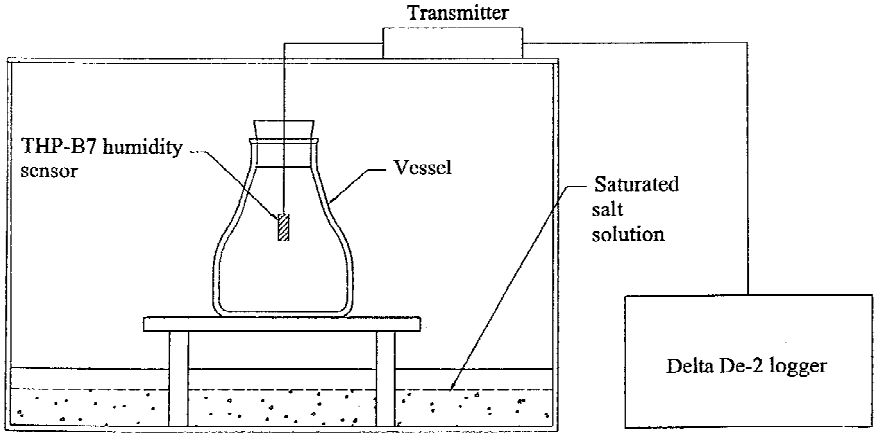

Figure 1. Schematic diagram of the air exchange rate measurement device by THP-B7 humidity sensor.

D. The vessel and the humidity sensor were placed in a plastic container, whose bottom had saturated salt solution ($MgCl_2 \cdot 6H_2O$). The outside environment of vessel was kept at 32.2% by this salt solution.
E. Because of the difference of the vapor concentration, the relative humidity of the vessel decrease, gradually.
F. Record measured data by the data logger. Collect and analyze these data pair (time and humidity) with the linear portion of the curve to calculate the K value.

2. Larger open area of vessels (Figure 2)

Because the opening of this type of vessel is larger than the size of HOBO Hygro-logger, this device can be put inside the vessel. The procedures for determination of K value are:

A. Add water into the vessel to maintain the vapor saturated condition as in the previous procedures A–C.
B. Start the operation of the HOBO Hygro-logger and put this device into the vessel; then cover the lid of vessel.
C. Place vessels into the plastic container. The saturated salt solution at the bottom maintains the constant relative humidity.
D. Sense and record the relative humidity of the air in the vessel, collect and analyze these data to calculate the K values.

Measurement of the effect of RH sensors

The effect of the sensor on the measurement of air exchange rate were studied by comparing the results of two types of vessels, French box and GA-7 box. There were five replicates for each treatment.

Table 1. Gas exchange rates for various vessels

	Vessel type	Exchange rates (h^{-1})
1.	Triangular glass flask	0.0145 ± 0.0025
2.	Japanese irregular box	0.0376 ± 0.0021
3.	French box	0.0333 ± 0.0018
4.	GA-7 box	0.0355 ± 0.0017
5.	Dutch box	0.0311 ± 0.0021

Mean $\pm$ SE.

Measurement of the influence of air current velocity

The experiment of setup to test the effect of air current on the air exchange rates is shown in Figure 3. The Dutch box vessel and DCB-1 vessel were used. The air velocity was measured with a hot wire anemometer. The range of the air current velocity was approximately 1.0–1.5 m s^{-1}.

Results

1. Gas exchange rates for several vessels

The typical curve of the relationship between relative humidity and time of the Japanese irregular box is shown in Figure 4. The gas exchange rates for six vessels are listed in Table 1. Most of the vessels had similar values, ranging from 0.03–0.041 h^{-1}, although the K value of the triangular glass flask was 0.0145 h^{-1}, significantly lower than other vessels.

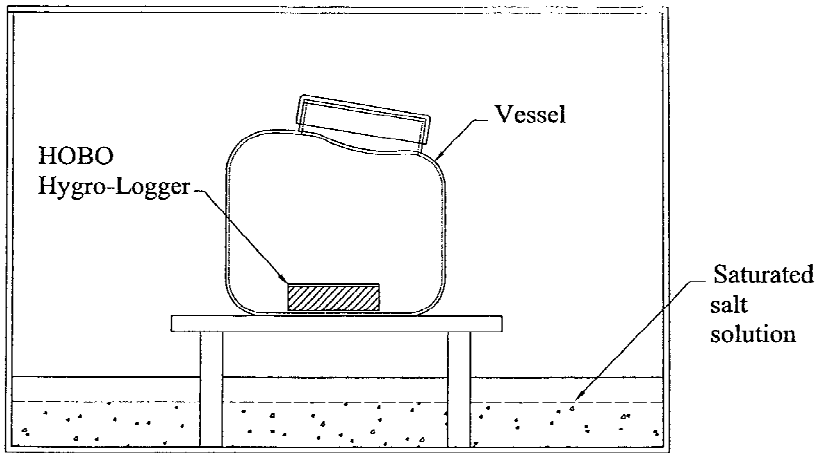

Figure 2. Schematic diagram of the air exchange rate measurement device by HOBO Hygro-Logger.

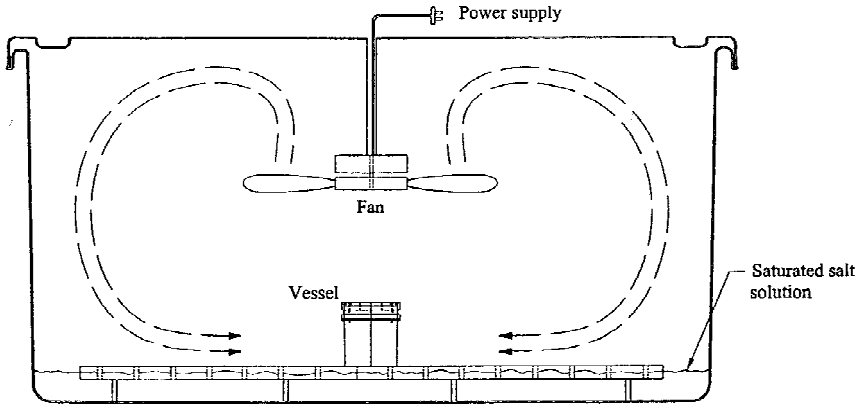

Figure 3. Experiment setup for the effects of air current on the air exchange rates of vessel.

Table 2. Number of gas exchange rates per hour for DCB-1 vessel box with different treatments

	Vessel type	Exchange rates (h^{-1})
A.	A hole inserted with cottons	0.0309 ± 0.0022
B.	A hole adhered with a layer of gas permeable tape	0.0310 ± 0.0018
C.	A hole adhered with two layers of gas permeable tape	0.0215 ± 0.0017
D.	A hole was inserted with cottons and adhered with a layer of gas permeable tape	0.0277 ± 0.0020

Mean $\pm$ SE.

2. Effect of different treatments on the gas exchange rates

The K values of the domestic DCB-1 vessel box with four different treatments are listed in Table 2. The vessel lid with a hole adhered with a layer of gas permeable tape had the largest K value. When two layers of gas permeable tape had been adhered to the hole, the K value decreased significantly.

3. Effect of air current velocities on the gas exchange rate

Table 3 shows the effects of air current on the gas exchange rates. The values of air exchange rates for vessels in fast air velocities were larger than those for vessels in stagnant air. The increases of exchange rates in fast air velocities were almost 1.6 times as large as those of vessels in stagnant air.

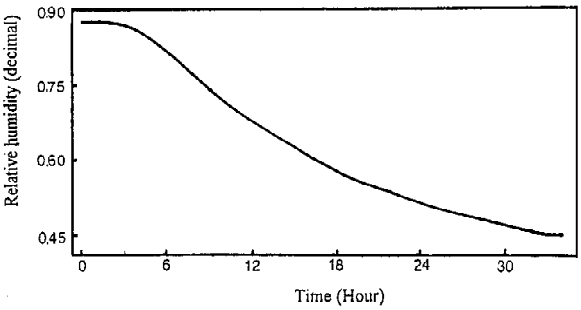

Figure 4. Relationship between relative humidity and time of Japanese irregular box vessel.

Table 3. Effect of air current on the gas exchange rates of tissue culture vessels

Vessels	Gas exchange rates (h^{-1})		
	Air near stagnant (A)	Forced air current (B)	B/A
Dutch type	0.0311 ± 0.0015	0.0511 ± 0.0021	1.64
DCB-1 box	0.0330 ± 0.0022	0.0454 ± 0.0018	1.38

Mean ± SE.

Discussion

Modify the microenvironment of vessels

In a plantlet micropropagation company, many sets of horizontal shelves are arranged in the culture room. Plantlet culture vessels are placed on the shelves. It is impractical to modify the microenvironment of each culture vessel with ventilation device. The adequate way to modify the microenvironment of vessels is to adjust the outside environment indirectly and to select vessels according to the required physical characteristic.

The air exchange rate of culture vessels influences the quantity of the air exchange between the inside and outside environment. Because the quantity of air exchange was small, the effect of air exchange rate of culture vessel on inside temperature was not significant (Chen, 2001). However, the gas concentrations in the vessel, i.e., CO_2 and C_2H_4, were affected by the gas concentration of outside air and the exchange rate of culture vessels.

Effect of gas exchange rate of culture vessels on the microenvironment

From the definition of gas exchange rate of culture vessels, the driving force for gas exchange was the different gas concentrations between inside and outside environment. The rate of gas exchange was influenced by the characteristics of culture vessels and ambient air condition. If the lid of vessel was sealed totally and no leakage could be found, the gas exchange rate of plantlet culture vessels was zero. In this case, inside gas concentrations; such as those of CO_2 and C_2H_4 cannot flow out, and may accumulate to the danger level. In order to avoid gas stress, the gas exchange rate of plantlet culture vessel could be modified by drilling a hole in the lid and closing this hole with gas permeable tape. If culture vessel has a larger gas exchange rate, more water will flow out from medium. The hardness of the medium increases as the moisture content decreases and the growth of plantlet may be retarded. Higher gas exchange rates also enhance the incidence of contamination.

The research group of Kozai et al. (1987) had proposed a CO_2 enrichment technique. Culture vessels are placed in the high CO_2 environment. Variation of the internal CO_2concentration can be estimated by the diffusion process that is described by Equation (2) of this study. To obtain the optimum gas concentration, $c(t)$ at time interval, t, researchers can control the gas concentration of the outside environment (C_{out}) and select an adequate culture vessel with the gas exchange rate of K_c. The measurement of gas exchange rates of various culture vessels is an essential for the microenvironment control of plantlet culture.

Ethylene has been found to affect the growth of plantlets in vessel (Matthys et al., 1996). Young (1985) illustrated the gas exchange method to remove ethylene. The ethylene concentration inside the vessel can be decreased by the gas exchange between inside microenvironment and outside air. Matthys et al. (1996) mentioned that the number of air exchange rates became more important but it was very difficult to control. From Equation (2), the C_{out} of ethylene can assume zero. C_i is the ethylene concentration produced by plantlets in vessel. $C(t)$ is the safe level for plantlets. T is the endurance period for plantlets. The characteristic of gas exchange rate of culture vessels, K_c, could be calculated by Equation (2), then be the criterion to adjust the gas exchange rate of culture vessel.

Application of the gas exchange rate on microenvironment control

The treatment of the layers of gas permeable tapes on the lid had been applied (Jeong et al., 1993). However, the effect of the gas permeable tapes on the gas exchange rates of vessels did not be quantified. The

results of this study indicates the effect of different treatments of gas permeable tapes on the gas exchange rates of vessels can be quantified easily.

Many culture vessels are placed on the shelves of culture room. The air velocity passed culture vessels are affected by the performance of air conditioner. The result of this study indicates the air velocity could enhance the gas exchange rate of culture vessel. However, more air exchange between inside and outside environment also increase the opportunity of contamination. This technique is safe to be applied for vessels were placed in the clean room or the gas permeable tape can keep out the microorganisms.

Measurement of gas exchange rate

Preparing some water inside culture vessel and weighting the vessel over a time-course seem to be a simple way to measure gas exchange rate of vessel. However, this method was limited by the accuracy of balance. For example, the data of a Dutch box vessel was follows: weights, 14.415 g; volume, 5.3268×10^{-4} m^3. As the air inside the vessel at 25°C and 30% RH, the weight of water vapor was 3.70349×10^{-3} g. The air inside the vessel at 25°C and 100% RH, the weight of water vapor was 1.2623×10^{-2} g. If the air relative humidity inside vessel was saturated, the total weight of vessel and air was 15.4276 g. As the air inside vessel was equilibrated with outside air at 25° and 30% RH, the total weight of vessel and air was 15.4187 g. The change of weight was 8.9195×10^{-3} g for several days. In the equilibrating period, the change of weight was so small, only a balance with very high accuracy performance can be adopted.

In this study, water vapor was adopted as trace gas. The change of relative humidity inside the vessel was measured continuously by a resistive relative humidity sensing element that had been calibrated. The outside environment was maintained at constant relative humidity by a saturated salt solution. The RH data were transformed into absolute humidity and the specific humidity ratio. The air exchange rates of several tissue culture vessels were then calculated. The effects of the air current velocities on the exchange rates of vessels were tested. This technique provides an inexpensive, rapid and simple way to determine the air exchange rate of culture vessel within a short period of time.

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