Technology & Product Reports

Carbon Dioxide Enrichment in Autotrophic Micropropagation: Methods and Advantages

Byoung Kyong Jeong¹,
Kazuhiro Fujiwara², and
Toyoki Kozai²

Additional index words. environmental control, autotrophy, CO₂ hazards, shoot induction

Summary. Autotrophic micropropagation has advantages over conventional micropropagation and can reduce costs of plantlet production. In this article, we describe advantages of autotrophic micropropagation and a practical and formulated method of enriching culture rooms with CO₂.

The goal of micropropagation is to mass-propagate genetically identical, physiologically uniform, and developmentally normal plantlets that can be acclimatized quickly and inexpensively. Development of both automated environmental control systems and improved in

vitro culture systems to enhance growth and development are essential for a significant reduction in production costs (Kozai and Jeong, 1993).

Recently, a novel micropropagation method, called autotrophic micropropagation, was proposed to improve growth and development of chlorophyllous shoots/plantlets ("plantlet" hereafter), by enhancing photosynthesis (Fujiwara et al., 1988; Kozai, 1991; Kozai et al., 1987, 1988; Kozai and Iwanami, 1988). An important component of the autotrophic micropropagation system is supplying the culture with CO₂; yet, to our knowledge, no detailed description of a successful method has been published.

In this article, the advantages of autotrophic micropropagation and parameters related to CO₂ enrichment of in vitro cultures are described.

Why enrich the culture room with CO₂?

Traditionally, explants and regenerated shoots in culture vessels have been considered to have little photosynthetic ability. On the assumption that plantlets require sugar in the culture medium as an energy and carbon source, plantlets have been cultured under predominantly heterotrophic (on artificially supplied carbon source) or mixotrophic (on both artificially supplied carbon source and photosynthetically produced carbon source) conditions.

However, recent research revealed that chlorophyllous plantlets had remarkable photosynthetic ability (Fujiwara et al., 1987; Kozai et al., 1987) and sometimes grew better under autotrophic rather than hetero- or mixotrophic condition when the physical and chemical culture environments were con-

trolled properly for photosynthesis [i.e., enriched CO₂ and elevated photosynthetic photon flux density (PPFD)] (Kozai and Iwanami, 1988; Kozai et al., 1988).

The CO₂ concentration in airtight vessels containing ornamental plantlets decreased to 70 to 80 ppm (v/v) within 2 to 3 h after the onset of the photoperiod (Fujiwara et al., 1987; Infante et al., 1989). The CO₂ concentration in those vessels was as low as the CO₂ compensation point of C₃ plants and was about 250 ppm (v/v) lower than the normal atmospheric CO₂ concentration [≈ 340 ppm, (v/v)].

Carbon dioxide enrichment under high PPFD (100 to 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was effective for promoting the growth of *Cymbidium* (Kozai et al., 1990), carnation (Kozai and Iwanami, 1988), and potato (Kozai et al., 1988) plantlets, regardless of the sugar content of the medium.

Autotrophic micropropagation has potential advantages over the conventional micropropagation method (Kozai and Jeong, 1993), including the following: 1) Enhanced growth and development of normal plantlets; 2) minimal use of growth regulators and other organic compounds; 3) possibility of using larger vessels with decreased incidence of biological contamination; 4) reduced loss of plantlets from biological contamination and from physiological, morphological, and genetic disorders; 5) simplified procedures for rooting and acclimatization; 6) automation, robotization, and computerization achievable; and 7) lower production costs.

Autotrophic micropropagation can be beneficial only for chlorophyllous cultures that show a positive CO₂ balance during the photoperiod. The use of a gas-permeable filter is expected to enhance uptake of mineral elements by plantlets as well because of increased transpiration.

How to enrich culture vessels with CO₂

Use of CO₂-permeable filter in the culture vessels and CO₂ enrichment in the culture room (indirect method). A few reports indicate a beneficial effect of a gas-permeable filter incorporated into the culture vessels under high PPFD on the net photosynthetic rate (NPR) and growth of plantlets in vitro (Kozai et al., 1988;

¹Department of Horticulture, Gyeongsang National University, Chinju Korea 660-701.

²Laboratory of Horticultural Engineering, Faculty of Horticulture, Chiba University, Matsudo, Chiba, Japan 271.

Kozai, 1991). Plantlets of some species derived from leafy single-node cuttings grew faster when cultured autotrophically in closed vessels containing a gas-permeable filter than when cultured heterotrophically in the conventional, relatively airtight, vessel (Kozai et al., 1988; Kozai, 1991). The percentage of vitrification decreases with the use of a gas-permeable filter, probably from a lowered relative humidity and increased gas exchange and dehydration of the medium.

Use of large culture vessels attached directly to a CO₂ supply system. Dry weight and the NPR of strawberry plantlets cultured on a sugar-free liquid medium were greater when cultured in a large vessel with a forced ventilation system, under a PPFD of 96 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, as compared to those of plantlets cultured using a conventional method (Fujiwara et al., 1988). However, forced ventilation with atmospheric air or a N₂-O₂-CO₂ mixture reduced propagule weight and shoot number in stage 2 *Rhododendron* cultured in vessels with 400 ml of air volume and under a PPFD of 39 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Walker et al., 1988). Such *Rhododendron* propagules were considered to have had low photosynthetic ability, and/or were apparently under a low PPFD and showed a negative CO₂ balance (releasing CO₂) during the photoperiod.

Parameters related to CO₂ enrichment in the culture room

Of the two methods of enriching CO₂ in the tissue culture systems described above, the rest of this paper focuses only on the indirect method. The discussion is limited to a situation with a steady state of CO₂ flow during the photoperiod. During the dark period, the CO₂ supply system simply can be shut off.

Parameters. To maintain CO₂ concentration in the vessel (C_{vessel}) at a desired level (500-800 $\mu\text{mol}\cdot\text{mol}^{-1}$), the preset CO₂ concentration in the room (C_{room}), CO₂ supply rate from a compressed CO₂ cylinder (C_{supply}) and number of air exchanges per hour of the vessel (N_{vessel}) can be determined from the following parameters (Fig. 1): CO₂ concentration outside the room (C_{out}), number of air exchanges per hour of the room (N_{room}), amount of air in the room (V_{room}), amount of

Fig. 1. Parameters related to CO₂ enrichment in the culture room. Abbreviations: C_{supply} -CO₂ supply rate from a CO₂ cylinder ($\mu\text{mol CO}_2/\text{h}$); C_{vessel} , C_{room} , and C_{out} -CO₂ concentration in the vessel, room, and outside of the room, respectively ($\mu\text{mol}\cdot\text{mol}^{-1}$); N_{vessel} and N_{room} -number of air exchanges per hour of the vessel and room, respectively (h); and V_{vessel} and V_{room} -volume of air in the vessel and room, respectively (mol).

air in the vessel (V_{vessel}), number of the vessels in the room (n), and the total NPR of the plantlet in the vessel.

Estimating the number of air exchanges per hour (N_{vessel}). In a steady state of CO₂ flow, the number of air exchanges per hour can be obtained from the following equation.

$$N_{\text{vessel}} = - \frac{1}{T} \frac{C_{\text{vessel}(t)} - C_{\text{room}}}{C_{\text{vessel}(0)} - C_{\text{room}}}$$

where $C_{\text{vessel}(0)}$ and $C_{\text{vessel}(t)}$ are the CO₂ concentrations in $\mu\text{mol}\cdot\text{mol}^{-1}$ in the room at time 0 and t , respectively; T , the time period between time 0 and t (h); and $\ln$, the natural logarithm. To determine the amount of CO₂ to be injected into the culture room, one can estimate the N_{vessel} containing no culture and medium by following the procedure described below:

1) Make a small hole (≈ 1 mm in diameter) on the vessel and seal the hole with a piece of adhesive tape; 2) replace the air in the vessel with the air containing CO₂ at concentrations higher than 10,000 ppm (v/v); 3) place the vessel on the culture bench and maintain a steady-state air movement [no CO₂ fluctuations around the vessel: $C_{\text{room}(0)}$ and C very close to each other]; 4) take air samples in the culture room and from the vessel with syringes by inserting the needle through the tape at two different times

(0 and t), at least 30 min apart; 5) measure CO₂ concentrations in the samples using gas chromatography, and plug the values into the equation described above. For C_{out} , use the average of the CO₂ concentrations in the room at 0 and t .

Relationship between the C_{room} , C_{supply} , and V_{room}

When photosynthesizing plantlets are present in the vessel, C_{vessel} decreases with time due to an increase in the NPR of the culture. The decrease in C_{vessel} can be compensated by supplying CO₂ with either one of the two methods described above. However, because it is difficult to increase N_{vessel} with time, except when a forced ventilation system is employed, C_{room} needs to be raised by increasing C_{supply} over time.

Fig. 2 shows the relationship between C_{room} , C_{supply} , and five different values of V_{room} , on the assumption that $N_{\text{room}} = 0.1 \text{ h}^{-1}$, $C_{\text{out}} = 350 \text{ mol}\cdot\text{mol}^{-1}$, and $n = 0$. When photosynthesizing cultures are present in the room, the $C_{\text{supply}} - C_{\text{room}}$ line will shift downward to a certain extent depending on the total NPR of the culture, and to the right to a certain extent as C_{room} increases. The photosynthetic rate of a single chlorophyllous shoot or plantlet in vitro of several species ranged from ≈ 1 to 10 $\mu\text{mol CO}_2/\text{h}$ at CO₂ concentrations of 100 to 500 $\mu\text{mol}\cdot\text{mol}^{-1}$ under conventional culture conditions

Fig. 2. Relationship between CO_2 concentration in the culture room (C_{room}), CO_2 supply rate from a CO_2 cylinder (C_{supply}), and amount of air in the room (V_{room}). The curves were obtained on the assumption that the number of air exchanges per hour of the vessel is 0.1 h and CO_2 concentration outside the room is $350 \mu\text{mol}\cdot\text{mol}^{-1}$.

(Fujiwara et al., 1987, 1988; Nakayama et al., 1991).

Is the CO_2 enrichment a health hazard?

The information on the allowable level CO_2 for human and cattle dwellings are described by Esmay and Dixon (1986). In the United States, the recommended maximum allowable CO_2 concentration in a building is 5000 ppm (v/v) or $9000 \mu\text{mol}\cdot\text{mol}^{-1}$. Carbon dioxide is not highly toxic in itself, but it can be responsible for O_2 deficiency or asphyxiation. Small increases above normal are quite harmless, but 10% (10,000 ppm, v/v) CO_2 can cause violent panting. Above this level, the effect is narcotic, even if there is adequate O_2 , because of the affinity of red blood cells for CO_2 over O_2 (Esmay and Dixon, 1986).

Carbon dioxide enrichment is a common practice in greenhouses for growing crops, and is generally successful economically up to 1000 to 1500 ppm (v/v) or $\mu\text{mol}\cdot\text{mol}^{-1}$ (Esmay and Dixon, 1986; Hickleton, 1988). Hickleton (1988), addressing specifically on the issue of the health hazards of the CO_2 enrichment of greenhouses, pointed out that CO_2 is a safe gas at concentrations generally encountered in CO_2 -enriched greenhouses (1000-2000 ppm, v/v) as long as the supply gas is void of other contaminant gases such as carbon monoxide.

Because the recommended optimum CO_2 concentration for culture vessels is around 500 to 800 ppm (v/v), and because the injection of the CO_2 gas in the culture room is only during the photoperiod, the potential health hazards of the gas to workers are thought to be quite small or negligible. While clinical studies have indicated minor physiological effects on human system of prolonged exposure to 1500 ppm (v/v) CO_2 , continuous daily exposure to that concentration is considered to be without hazard in the work environment (Hickleton, 1988). However, a possible health risk due to the continued exposure for 8 h/day and 5 days/week to CO_2 at concentrations higher than the maximum allowable levels, which is necessary only when the N_{vessel} is extremely low, should be realized. Therefore, the use of a gas-permeable membrane filters with an appropriate surface area on vessels is recommended as a method to enhance gas exchange between the culture vessels and the room, and to lower the CO_2 concentration necessary to enrich the room.

Literature Cited

Esmay, M.L. and J.E. Dixon. 1986. Environmental control for agricultural buildings. AVI, Westport, Conn. p. 166-167, 256.

Fujiwara, K., T. Kozai, and I. Watanabe.

1987. Measurements of carbon dioxide gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets. J. Agr. Meteorol. 43:21-30.

Fujiwara, K., T. Kozai, and I. Watanabe. 1988. Development of a photoautotrophic tissue culture system for shoots and/or plantlets at rooting and acclimatization stages. Acta Hort. 23:153-158.

Hickleton, P.R. 1988. CO_2 enrichment in the greenhouse. Timber Press, Portland, Ore.

Infante, R., E. Magnanini, and B. Righetti. 1989. The role of light and CO_2 in optimizing the conditions for shoot proliferation of *Actinidia deliciosa* in vitro. Physiol. Plant. 77:191-195.

Kozai, T., Y. Iwanami, and K. Fujiwara. 1987. Effect of CO_2 enrichment on the plantlet growth during the multiplication stage. Plant Tissue Cult. Lett. 4:22-26.

Kozai, T. and Y. Iwanami. 1988. Effects of CO_2 enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during the preparation stage. J. Jpn. Soc. Hort. Sci. 57:279-288.

Kozai, T., Y. Koyama, and I. Watanabe. 1988. Multiplication of potato plantlets in vitro with sugar free medium under high photosynthetic photon flux. Acta Hort. 230:121-127.

Kozai, T., H. Oki, and K. Fujiwara. 1990. Photosynthetic characteristics of *Cymbidium* plantlet in vitro. Plant Cell, Tissue Organ Cult. 22:205-211.

Kozai, T. 1991. Autotrophic micropropagation, p. 313-343. In: Y.P.S. Bajaj (ed.). Biotechnology in agriculture and forestry 17: High-tech and micropropagation I. Springer-Verlag, New York.

Kozai, T. and B.R. Jeong. 1993. Environmental control in plant tissue culture and its application for micropropagation, p. 95-116. In: Y. Hashimoto, G.P.A. Bot, W. Day, H.-J. Tantau, and H. Nonami (eds.). The computerized greenhouse: Automatic control application in plant production. Academic, New York.

Nakayama, M., T. Kozai and K. Watanabe. 1991. Effect of presence/absence of sugar in the medium and natural/forced ventilation on the net photosynthetic rates of potato explants in vitro. Plant Tissue Cult. Lett. 8(2):105-109.

Walker, P.N., C.W. Heuser, and P.H. Heinemann. 1988. Micropropagation: Studies of gaseous environments. Acta Hort. 230:145-151.