Journal of Plant Biology, June 2001, 44(2) : 87-91

CO_2-Enrichment and Photosynthetic Photon Flux Affect the Growth of in Vitro-Cultured Apple Plantlets

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Micro-cuttings (shoots with two small leaves) of cultivar M9 apple were cultured in-vitro for 40 d under CO_2-enriched and non-enriched (i.e., ambient air) conditions, and at a PPF of 40 or 100 μmol m^{-2} s^{-1}. Afterward, shoot length, number of leaves, leaf area, chlorophyll content, shoot and root fresh weights, and % survival were recorded. Those plantlets grown under CO_2- and PPF-enriched treatments were healthy and vigorous, and showed higher values for their growth parameters. In contrast, those grown without supplemental CO_2 or PPF often showed hyperhydricity. We also demonstrated that CO_2 enrichment and a relatively high PPF during in-vitro culture promoted normal photosynthesis and growth after ex-vitro transplantation.

Keywords: Carbon dioxide, in-vitro culture, *Malus domestica*

In vitro-raised plantlets often show poor performance after ex-vitro transplantation. This may result from low photosynthetic rates of the plantlets during the culturing period, incomplete autotrophy, high transpiration rates because of thin cuticle layers and abnormal stomatal functioning, incomplete rooting, or physiological disorders such as hyperhydricity (Kozai, 1991). However, the photosynthetic capability of plantlets developed in-vitro can be similar to that of plants grown ex-vitro. The former may even develop autotrophy if the in-vitro environmental factors, such as CO_2 and light (i.e., photosynthetic photon flux; PPF) are properly controlled (Kozai et al., 1987, 1988; Kozai and Iwanami, 1988).

Apple plants regenerated in-vitro do not adapt easily to greenhouse conditions compared with other fruit crops because their leaves desiccate very rapidly when the plants are removed from the culture container. These plants often display abnormalities such as hyperhydricity (Zimmerman, 1984). In this study, we investigated in vitro-raised apple shoots that were cultured in-vitro for rooting under CO_2-enriched and high PPF conditions. Our major objectives were to determine 1) the effects of photosynthetic photon flux (PPF) and CO_2-enrichment on in-vitro growth during the rooting stage and 2) the contribution of these factors to the development of healthy plantlets after transplantation.

MATERIALS AND METHODS

Culture of Plant Material

Culture conditions are generally described in Table 1. Explants (shoots with two small leaves) were excised, at the late-multiplication stage, from in vitro-grown apple [*Malus domestica* cultivar M9 (EMLA-9)]. These plantlets had been cultured under conventional conditions. Explants developed on media in 100-mL culture flasks. The mouths of the flasks were wrapped with polypropylene film, with or without a membrane filter at the center of the opening.

Treatments

One treatment involved culturing under normal conditions, using CO_2 non-enriched (i.e., ambient) air and one of two PPF levels (40 or 100 μmol m^{-2} s^{-1}). In this treatment, the mouths of the flasks were covered with polypropylene film (0.01 to 0.04 air exchanges per hour; Fujiwara et al., 1987).

The second treatment included CO_2-enriched air and either 40 or 100 μmol m^{-2} s^{-1} PPF. Here, the

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Abbreviations: MS, Murashige and Skoog medium, PPF, Photosynthetic photon flux, IBA, Indolebutyric acid.

Table 1. Description of culture conditions.

Plant material
Species: Apple, *M. domestica* cultivar M9
Explant: Micro-cuttings-each shoot with two small leaves
Fresh weight: 30 mg/explant
Plant density: 3 explants/vessel
No. of plantlets per treatment: 30
Culture vessel
Type: Conical flask (Corning Co., USA)
Volume: 100 mL; mouth of the flask covered with polypropylene film in CO_2 non-enriched treatment; cover with membrane filter in the center (10 mm in diameter) in CO_2-enriched treatment.
No. of air exchanges: 3.4 h^{-1} (CO_2-enriched conditions). 0.01 to 0.04 h^{-1} (CO_2 non-enriched conditions).
Growth medium
Mineral composition: Half-strength Murashige and Skoog (1962) medium supplemented with 1.0 mg L^{-1} IBA, 200 mg L^{-1} activated charcoal (Sigma, USA), and 30 g L^{-1} sucrose
Amount: 50 mL/vessel, Gelling agent: Gelrite-2.4 g L^{-1}.
pH: 5.8 before autoclaving.
Culture conditions
PPF: 40 or 100 µmol m^{-2} s^{-1}.
CO_2 concentration: 1000 µmol mol^{-1} under for CO_2-enriched treatment, or ambient air for control treatment.
Relative humidity: 70%.
Air temperature: 25 ± 1°C.
Photoperiod: 16 h d^{-1}, 24-h cycle.
Culture period: 40 d.

mouths of the flasks also were covered with polypropylene film, but with one 10-mm-diameter membrane filter (Santomi Sangyo Co. Ltd., Japan) added in the center. number of air exchanges was). The number of air exchanges (3.4 h^{-1}) in each vessel was calculated as the hourly ventilation rate divided by the air volume of the vessel, according to the method and equation of Fujiwara et al. (1987) and Kozai et al. (1986). Each PPF treatment was maintained in separate culture chambers one supplemented with 1000 µmol mol^{-1} of CO_2, the other without an external CO_2 supply. These concentrations were maintained with an infrared CO_2 controller (Dasol Scientific Co. Ltd., Korea); liquid CO_2 was supplied by Praxair Co. Ltd., Korea.

Measurements

After 40 d of culture, plantlets from each treatment were harvested for measuring shoot length, number of leaves per plantlet, as well as shoot and root fresh weights. Chlorophyll content was determined with a chlorophyll meter (SPAD-502; Minolta Camera Co. Ltd., Japan). Leaf surface area was measured with a leaf-area analysis instrument (SKYE industries Ltd., UK). All data for these growth parameters were analyzed from ten replicates; mean values were compared by Duncan's Multiple Range Test.

Scanning Electron Microscopy

Specimens for scanning electron microscopy (SEM) were prepared according to the procedure of Donnelly et al. (1987). Leaf sections were fixed with 4% glutaraldehyde in a cacodylate buffer (pH 7.0) for at least 2 h; washed in dH_20; soaked for 1.5 h with 1% osmium in a 0.1 M cacodylate buffer (pH 7.0); washed again in dH_20; and dehydrated through an ethanol series. Leaf sections were critical-point-dried in CO_2, mounted on aluminum stubs with silver-epoxy paste, sputter-coated with gold, and examined and photographed in a Hitachi - S2300 SEM.

Ex-Vitro Acclimatization of Plantlets

Plantlets from each treatment were transplanted into trays containing vermiculite and perlite (1:1) and grown under greenhouse conditions of 50% relative humidity; day/night temperatures of 25/15°C, and a 14-h photoperiod, with PPF of 500 µmol m^{-2} s^{-1}. Their percent survival rate was determined 30 d after transplantation.

RESULS AND DISCUSSION

Growth and Development

Compared with the non-enriched controls, plantlets grown under CO_2-enriched conditions had greater shoot lengths, numbers of leaves per plantlet, leaf areas, and shoot and root fresh weights (Table 2). The optimal PPF under these conditions was 100 µmol m^{-2} s^{-1}. Overall, shoot and root fresh weights as well as leaf area increased by two-fold when plants were grown under CO_2- and PPF-enriched conditions. Similar growth responses to in-vitro carbon dioxide and light enrichment have been reported by Kozai et al. (1987), Cournac et al. (1991), Fujiwara et al. (1992), Buddendorf-Joosten and Woltering (1996), and Tisserat et al. (1997). In the current study, chlorophyll content also was higher for plants grown under CO_2- and PPF-enriched conditions. This result was also observed in potato plants (Buddendorf-Joosten and

Table 2. Growth parameters of apple M9 plantlets cultured in-vitro under CO_2-enriched and non-enriched conditions; data collected after 40 d of culture.

CO_2 ($\mu mol\ mol^{-1}$)	PPF ($\mu mol\ m^{-2}\ s^{-1}$)	Shoot length* (cm)	No. of leaves*	Leaf area* (cm^2)	Shoot fresh weight* (mg)	Root fresh weight* (mg)	Chlorophyll content*[z] (SPAD values)
Ambient air	40	1.1b	5.3b	2.2b	46.2d	27.3c	22.1c
	100	1.4ab	5.5b	2.4b	70.4c	244.4bc	31.4b
1000	40	1.5a	7.5a	2.5n	101.3n	422.3b	35.3a
	100	1.5a	7.7a	4.9a	147.4a	655.3a	31.2b

*Mean values followed by different letters within each column differ significantly according to Duncan's multiple range test at $p < 0.05$.
[z]Measured by Minolta SPAD-502 Chlorophyll Meter.

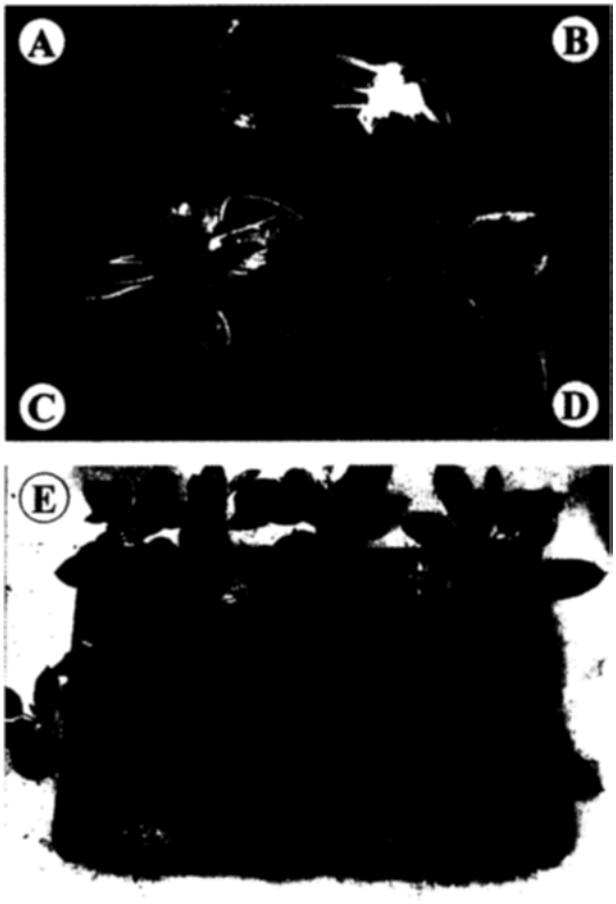

Figure 1. In vitro-grown apple plantlets under CO_2-enriched or non-enriched treatments, and at PPF of 40 or 100 $\mu mol\ m^{-2}\ s^{-1}$ (after 40 d of culture). **A.** Plantlet grown under CO_2 non-enriched (ambient air) condition and PPF of 40 $\mu mol\ m^{-2}\ s^{-1}$. **B.** Plantlet grown under CO_2 non-enriched (ambient air) condition and PPF of 100 $\mu mol\ m^{-2}\ s^{-1}$. **C.** Plantlet grown under CO_2-enriched (1000 $\mu mol\ mol^{-1}$) condition and PPF of 40 $\mu mol\ m^{-2}\ s^{-1}$. **D.** Plantlet grown under CO_2-enriched (1000 $\mu mol\ mol^{-1}$) condition and PPF of 100 $\mu mol\ m^{-2}\ s^{-1}$. **E.** Acclimatized plants (30 d after transplantation).

Woltering, 1996).

Plantlets cultured under CO_2 non-enriched conditions often had glossy and vitreous (hyperhydric) leaves (Fig. 1, A and B). However, those grown with supplemental CO_2 were healthy and vigorous (Fig. 1, C and D). Stomata that differentiate on in-vitro leaves usually vary in shape and tend to persist in an open state (Wetzstein and Sommer, 1983; Blanke and Belcher, 1989; Zacchini et al., 1997). This open state probably generally results from stomata that collapse because of the elevated relative humidity in the culture vessel (Brainerd and Fuchigami, 1982; Wardle and Short, 1983).

SEM showed that the abaxial stomata were more numerous (400 to 430 stomata per mm^2) under CO_2 non-enriched conditions (0.01- 0.04 h^{-1} air exchanges) compared with 200 to 250 stomata per mm^2 for enriched leaves (3.4 h^{-1} air exchanges; Fig. 2, A and B). Stomata also were often deformed on plantlets grown in the CO_2 and PPF non-enriched treatments. Increased CO_2 concentrations during in-vitro development also caused significant decreases in stomatal density in grape and coffee (Ross-Karstens et al., 1988). Therefore, in vitro-cultured plantlets can show normal photosynthesis and growth patterns if their environment is supplemented with enhanced PPF and CO_2, along with an increased number of air exchanges. These results agree with those of Desjardins et al. (1988), Fujiwara et al. (1988), Kozai et al. (1988), Cournac et al. (1991), Figueira et al. (1991), Fujiwara and Kozai (1995), Jeong et al. (1995), and Tisserat et al. (1997).

Ex-Vitro Survival of Plantlets

In vitro-grown plantlets were transplanted ex-vitro. After 30 d, the survival rate was 30 to 50% for plantlets that had been grown under conventional in-vitro

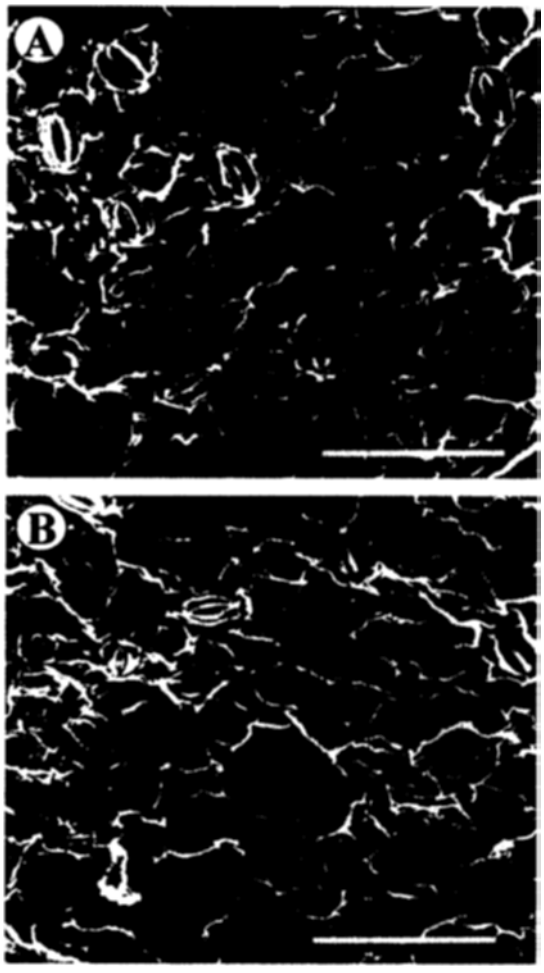

Figure 2. Scanning electron micrographs of abaxial leaf surface of tissue-cultured apple plantlets, showing stomatal characteristics. **A.** Leaf surface of plantlet grown under CO_2-enriched (1000 μmol mol^{-1}) condition and PPF of 100 μmol m^{-2} s^{-1}. **B.** Leaf surface of plantlet grown under CO_2 non-enriched (ambient air) condition and PPF of 40 μmol m^{-2} s^{-1} (note the greater number of stomata, some deformed).

Table 3. Percent survival of in vitro-grown apple plantlets (raised in-vitro under CO_2-enriched and non-enriched treatments, and at different PPF levels) 30 d after transplantation ex vitro.

CO_2 (μmol m^{-2} s^{-1})	PPF (μmol mol^{-1})	% Survival
Ambient air	40	30
	100	50
1000	40	65
	100	80

conditions (i.e., CO_2 non-enriched). This was lower than the 65 to 80% for plantlets that had developed under CO_2- and PPF-enriched conditions and that were now well established (Table 3; Fig. 1E). This suggests that controlling the microenvironment during in-vitro propagation may reduce the physiological stresses that usually accompany the transfer of plantlets to soil. Improving the process of acclimatization should, therefore, increase survival rates and enable the in-vitro foliage to persist and contribute to better plantlet establishment.

ACKNOWLEDGEMENS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for Development of Advanced Horticultural Technology at Chungbuk National University.

Received April 6, 2001; accepted May 7, 2001.

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