A Forced Ventilation Micropropagation System for Photoautotrophic Production of Sweetpotato Plug Plantlets in a Scaled-up Culture Vessel: I. Growth and Uniformity

Jeongwook Heo,[1]
Sandra B. Wilson,[2] and
Toyoki Kozai[3]

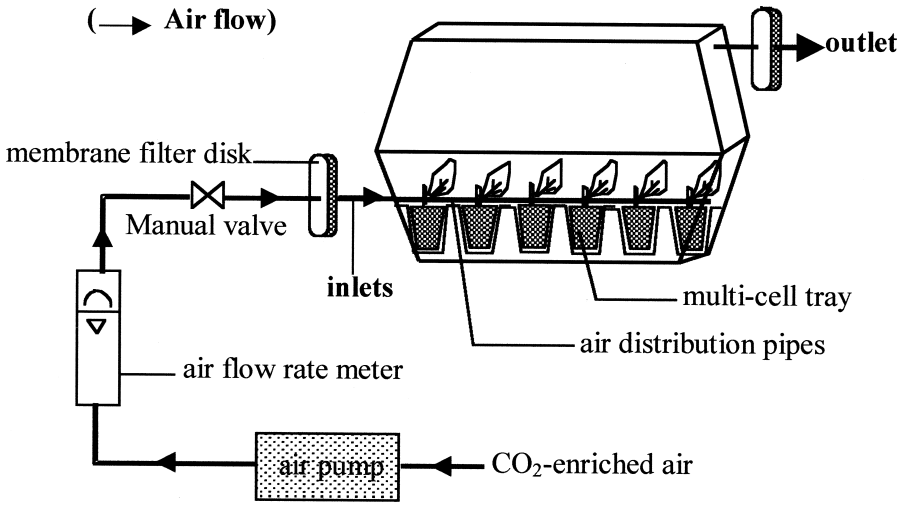
ADDITIONAL INDEX WORDS. **Air currents, air distribution system, culture vessel, *Ipomoea batatas*, plant tissue culture, photoautotrophic culture, photomixotrophic culture**

SUMMARY. **An improved forced ventilation micropropagation system was designed with air distribution pipes for uniform spatial distributions of carbon dioxide (CO_2) concentration and other environmental factors to enhance photoautotrophic growth and uniformity of plug plantlets. Single-node stem cuttings of sweetpotato [*Ipomoea batatas* (L.) Lam. 'Beniazuma'] were photoautotrophically (no sugar in the culture medium) cultured on a mixture of vermiculite and cellulose fibers with half-strength Murashige and Skoog basal salts in a scaled-up culture vessel with an inside volume of 11 L (2.9 gal). CO_2 concentration of the supplied air and photosynthetic photon flux on the culture shelf were maintained at 1500 μmol·mol^{-1} and 150 μmol·m^{-2}·s^{-1}, respectively. Plantlets grown in forced ventilation systems were compared to plantlets grown in standard (natural ventilation rate) tissue culture vessels. The forced (F) ventilation treatments were designated high (FH), medium (FM), and low (FL), and corresponded to ventilation rates of 23 mL·s^{-1} (1.40 inch^3/s), 17 mL·s^{-1} (1.04 inch^3/s), and 10 mL·s^{-1} (0.61 inch^3/s), respectively, on day 12. The natural (N) ventilation treatment was extremely low (NE) at 0.4 mL·s^{-1} (0.02 inch^3/s), relative to the forced ventilation treatments. On day 12, the photoautotrophic growth of plantlets was nearly two times greater with the forced ventilation system than with the natural ventilation system. Plantlet growth did not significantly differ among the forced ventilation rates tested. The uniformity of the plantlet growth in the scaled-up culture vessel was enhanced by use of air distribution pipes that decreased the difference in CO_2 concentration between the air inlets and the air outlet.**

In conventional micropropagation, chlorophyllous shoots and plantlets are cultured on a medium containing sugar (i.e., photomixotrophically) using a relatively small culture vessel (several hundred milliliter in volume), at a low photosynthetic photon flux (PPF). The culture vessel closures are designed to prevent the entering of microbes, but, consequently, restrict the gas exchange between the inside and outside of the culture vessel (Desjardins, 1995). CO_2 concentration in the vessel containing plantlets decreases rapidly to 50 to 100 μmol·mol^{-1} after 1 to 2 h of the start of a photoperiod due to the limited gas exchange (Kozai, 1991). Fujiwara et al. (1987) showed that the low net photosynthetic rate and poor growth of in vitro plantlets were due to the low CO_2 concentration inside the vessel during the photoperiod. In addition, the air speed around in vitro plantlets in an airtight culture vessel is substantially lower than that in the greenhouse or field (Kitaya et al., 1997), which reduces the photosynthesis and transpiration of plantlets and thus their growth in vitro (Kozai et al., 1992).

Zobayed et al. (1999) engineered a forced ventilation system that supplies sterile nutrient solution throughout an extended culture period. They found that a stagnant nutrient solution under photoautotrophic forced ventilation conditions significantly increased the growth and net photosynthetic performance of in vitro plantlets. Heo and Kozai (1999) utilized a simplified, commercially available, forced ventilation micropropagation system using a scaled-up [12.8 L (3.4 gal)] culture vessel that was 25 to 40 times larger than typically used. With forced ventilation using an air pump, the photoautotrophic growth and net photosynthetic rate of sweetpotato plug plantlets were remarkably in-

Fig. 1. Component diagram of a forced ventilation micropropagation system.



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[1]Principal researcher, Research Center for the Development of Advanced Horticultural Technology, Department of Horticulture, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea.

[2]Assistant professor, corresponding author, Department of Environmental Horticulture, Indian River Research and Education Center, University of Florida, 2199 South Rock Road, Fort Pierce, FL 34945.

[3]Professor, Department of Bioproduction Science, Faculty of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan.

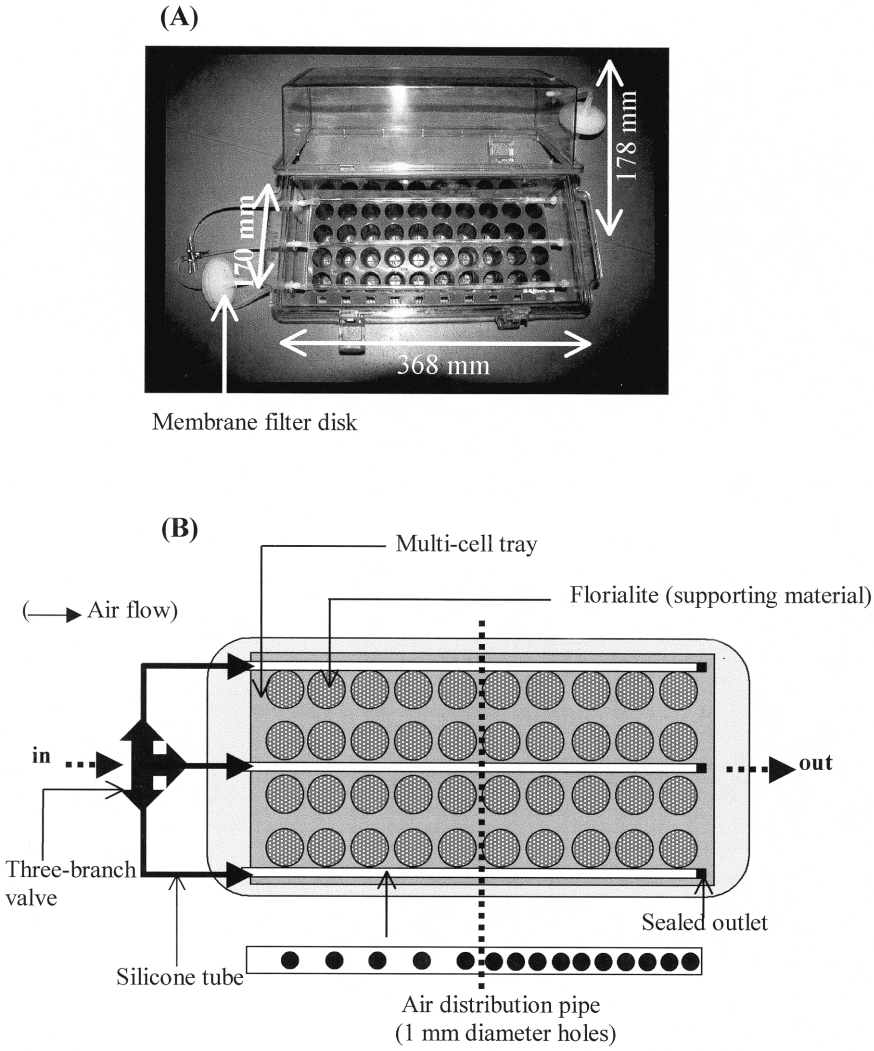

Fig. 2. Photograph (A) and top-view schematic diagram (B) of the culture vessel with air distribution pipes in the forced ventilation micropropagation system; 25.4 mm = 1.0 inch.

creased, compared with those under the conventional, natural ventilation conditions. The plantlet growth, however, was not spatially uniform in the force-ventilated culture vessel, as growth was greater near the air inlet than near the air outlet. Nonuniform spatial distributions of air velocity and CO_2 within the culture vessel caused significant spatial variations of the plantlet growth in the scaled-up culture vessel.

The objective of this study was to develop an improved forced ventilation micropropagation system (uniform air velocity and CO_2 distribution) for enhancing the growth rate and final uniformity of sweetpotato plug plantlets by using air distribution pipes in the culture vessel.

Materials and methods

PLANT MATERIAL AND CULTURE CONDITIONS. Single-node stem cuttings (initial fresh weight: 80 ± 23 mg), each with a single leaf excised from in vitro sweetpotato plantlets, were used as explants in all treatments. A mixture of vermiculite and cellulose fibers (Florialite, Nisshinbo Industries, Inc., Tokyo, Japan) was used as supporting material for plantlets; 30 mL (1.0 fl oz) per explant of half strength MS (Murashige and Skoog, 1962) basic components, without sucrose, was added to each culture vessel. The pH of culture medium was adjusted to 5.7 before autoclaving.

Culture vessels were placed for 12 d in a culture room, where room air temperature and relatively humidity were maintained at 29/26 ± 0.5 °C (84/79 ± 2 °F) (photo/dark period) and 80% ± 5%, respectively. A photoperiod of 16 h was provided by cool-white fluorescent lamps at 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPF. Photosynthetic photon flux was measured at 20 locations on the empty culture shelf to ensure light uniformity in all vessels. The atmospheric CO_2 concentration in the culture room was controlled at 1500 $\mu mol \cdot mol^{-1}$ with an infrared CO_2 controller (ZFP, Fuji Electric Co. Ltd., Tokyo, Japan) and recorded by a computerized system (Greenkit 100, E.S.D. Co. Ltd., Tokyo, Japan).

FORCED VENTILATION MICROPROPAGATION SYSTEM. The forced ventilation micropropagation system included acrylic air distribution pipes within a scaled-up culture vessel (Bio-Safe Carrier box, Nalge Co. Ltd.) 368 mm (14.5 inches) long by 178 mm (7 inches) high by 170 mm (6.7 inches) wide, 11.14 L (2.94 gal) air volume), an air flow rate meter (Purge type: No. 5S, Kofloc Co. Ltd., Tokyo, Japan) with a manual air valve (KT-6, Iuchi Sangyo Co. Ltd., Tokyo, Japan), membrane filter disks, and an air pump for supplying the CO_2-enriched air (Fig. 1). The culture vessel contained a 4 by 10-cell tray (Minoru Sangyo Co. Ltd., Tokyo, Japan), three air inlets, and one air outlet for forced ventilation. Membrane filter disks (Nippon Millipore Co. Ltd., Yonezawa, Japan, 0.5 μm) were attached to each of the inlets and outlets to prevent microbes from entering. To distribute the ventilated air evenly in the culture vessel, three acrylic air distribution pipes [3-mm (0.08-inch) inside diameter] with 1-mm (0.04-inch) diameter holes were set on the surface of the multicell tray containing the supporting material (Fig. 2). To create uniform airflow along the length of the pipes, the three distribution pipes were designed exactly the same, whereby twice as many holes were inserted near the air outlet than near the inlet. The end of each air distribution pipe was sealed so that the ventilation air entered the vessel throughout the numerous pipe holes and exited the vessel through the outlet hole made on the vessel wall (opposite side of the inlet). The three air inlet pipes and one air outlet in the culture vessel were located at opposite ends of the vessel at heights of 40 and 168 mm (1.6 and 6.6 inches) from the bottom of the vessel, respectively. The three-branch valve (Fig. 2B) was used to distribute the inlet air among the three pipes. The forced ventilation rate was measured with an air flow rate meter and controlled with a flow rate controller. The average air speed was cal-

Table 1. Ventilation rate and average air speed on day 12 within forced ventilation and natural ventilation systems; FH = high forced ventilation, FM = medium forced ventilation, FL = low forced ventilation, and NE = extremely low natural ventilation.

Treatment code	Ventilation rate ($mL \cdot s^{-1}$)[z]	Average air speed ($cm \cdot s^{-1}$)[y]
FH (forced, high)	23.0	0.08
FM (forced, medium)	17.0	0.06
FL (forced, low)	10.0	0.03
NE (natural, extremely low)	0.4	not available

[z]16.4 $mL \cdot s^{-1}$ = 1.0 $inch^3/s$.
[y]1.00 $cm \cdot s^{-1}$ = 0.39 inch/s.

culated by dividing the forced ventilation rate by the cross sectional area of the vessel.

NATURAL VENTILATION MICROPROPAGATION SYSTEM. Three cylindrical polycarbonate boxes with dimensions of 10 cm (3.9 inches) high by 8 cm (3.2 inches) wide and inside volume of 480 mL (29 $inches^3$) (Biopot, Nicca Chemical Co. Ltd., Japan) were used as culture vessels for the natural ventilation (more conventional) treatment. To increase the number of natural air exchanges of the culture vessel, a membrane filter disk (Milli-Seal, Millipore Co. Ltd., Japan, pore size: 0.5 μm) was attached over each of three 10-mm (0.39-inch) diameter holes on the lid of the vessel. The air exchange rate of the culture vessels was estimated to be 3.0/h according to the method described by Kozai et al. (1986). Consequently the ventilation rate was approximately 0.4 $mL \cdot s^{-1}$.

TREATMENTS. Ventilation treatments were designed to supply forced air with a controlled amount of CO_2 at varying rates to determine the best spatial uniformity of gas distribution within the vessels. Plantlets grown in forced ventilation systems were also compared to plantlets grown in standard (natural ventilation rate) tissue culture vessels (Table 1). The forced (F) ventilation treatments were designated high (FH), medium (FM), and low (FL). The natural (N) ventilation treatment was extremely low (NE), relative to the forced ventilation treatments. In FH, FM, and FL treatments, the forced ventilation rates in the vessels were gradually increased with passage of days during the culture period. The ventilation rates on day 12 were set at 23, 17, and 10 $mL \cdot s^{-1}$, respectively. Forty explants were planted in each tray of FH, FM and FL treatments. Because of space limitations, only one tray was used per treatment. In the NE treatment, the standard practice of planting four explants in each of the three cylindrical, polycarbonate culture vessels was used.

VISUALIZATION OF AIR CURRENT IN A SCALED-UP CULTURE VESSEL. The air current inside the culture vessel with sweetpotato plantlets was visualized at the ventilation rate of 13 $mL \cdot s^{-1}$ (0.8 $inch^3$/s) using fine particles of metaldehyde ($(C_2H_4O)_4$) as tracers and a high resolution and contrast camera (Super-eye C2874, Hamamatsu Photonics, Tokyo, Japan) as described by Kitaya et al. (1997).

MEASUREMENTS OF THE PLANTLET GROWTH AND CO_2 CONCENTRATION. Sweetpotato plantlets were harvested, and destructive measurements were taken on fresh and dry weights on day 12. Leaf area per plantlet was estimated by a leaf area meter (Fujitsu Kyusyu System Engineering Co. Ltd., Tokyo, Japan).

Two holes (2 mm diameter) were made at the side-wall of the scaled-up culture vessel for measuring the CO_2 concentrations near the air inlets and outlets. The holes were located at 90 and 278 mm (3.5 and 10.9 inches), respectively, from the air inlets along the longitudinal direction of the culture vessel. The CO_2 concentration was measured in each treatment during the photoperiod when CO_2 concentration between inside and outside of the culture vessel was stable over time. The CO_2 concentration measurement was conducted by using a gas chromatograph (GC9A, Shimadzu Corporation, Kyoto, Japan).

STATISTICAL ANALYSIS. The nature of the experimental design did not allow for randomized vessels or an equal number of samples between treatments, therefore, standard deviations (σ) were calculated among treatments and used to detect differences between treatments at 95% confidence.

Results and discussion

GROWTH OF SWEETPOTATO PLUG PLANTLETS. Photographs of sweetpotato plantlets on day 12 are shown in Fig. 3. The fresh weight per plantlet on day 12 was more than 1.2 times greater in FH, FM, and FL treatments than in NE treatment (Table 2). The dry weight per plantlet was 1.4 to 1.5 times greater in the forced ventilated treatments than in the NE. Final plantlet fresh and dry weights were similar within FH, FM, or FL ventilation treatments.

Leaf area per plantlet was 1.7 times greater in FH and FM than in NE. There was no difference in leaf area per plantlet among the FH, FM and FL treatments. It has been reported earlier that leaf area per plantlet of sweetpotato was significantly greater in a forced ventilation micropropagation system, under high CO_2 concentration and high PPF culture conditions, than in a natural ventilation micropropagation system (Heo and Kozai, 1999). A similar result has been shown for sweetpotato plantlets cultured photoautotrophically in vitro using vermiculite in the culture vessel with a high number of natural air exchanges (7.4/h) (Kozai et al., 1996). The benefit of forced ventilation for promotion in photosynthesis and growth also has been shown by De et al. (1993). They showed that the growth of shoot and root in geranium plantlets was two-three times greater under forced ventilation [air flow rate of 6 $L \cdot h^{-1}$ (1.6 gal/h)] than under the no ventilation condition. The thickness of the boundary layer around the leaves may have decreased by increasing the air current speed (Yabuki and Miyakawa, 1970), thereby reducing the resistance to CO_2 diffusion from air to leaf surface and promoting growth.

SPATIAL VARIATIONS IN CO_2 CONCENTRATION AND PLANTLET GROWTH INSIDE THE CULTURE VESSEL. Throughout the culture period, the CO_2 concentration inside the culture vessel during the photoperiod was more than 350 $\mu mol \cdot mol^{-1}$ lower in NE than in FH, FM, and FL treatments (Fig. 4). This correlated with a net photosynthetic rate (NPR) per plantlet on day 12 that was almost five times higher in the FH treatment than in the NE treatment (Wilson et al., 2000). There were no significant differences between the FH

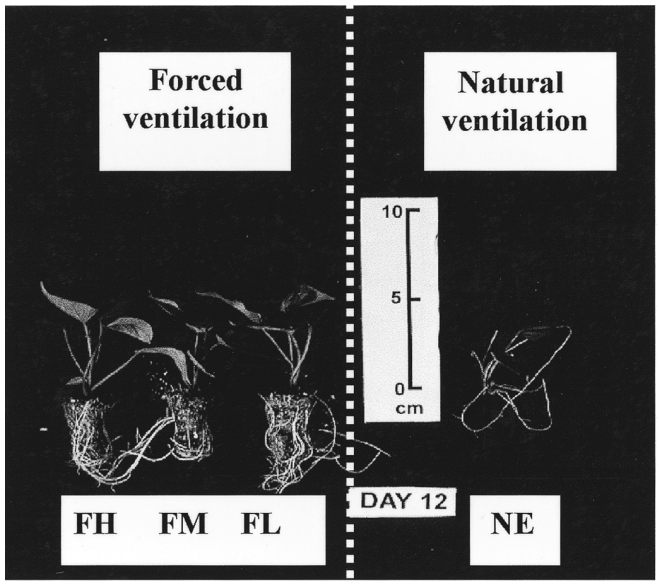

Fig. 3. Photograph of sweetpotato plug plantlets on day 12; FH = high forced ventilation, FM = medium forced ventilation, FL = low forced ventilation, and NE = extremely low natural ventilation; 2.54 cm = 1.0 inch.

and FM treatments in CO_2 concentrations between the air inlets and the air outlet (Fig. 4). In the FL treatment, however, CO_2 concentration at the air outlet was about 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ lower than that at the air inlets.

The spatial variations in plantlet growth between the air inlets and the air outlets were less uniform in FL than in FH and FM (data not presented) due to the differences in the forced ventilation rate, air speed and resulting uneven distribution of CO_2 concentration inside the culture vessel during the photoperiod. The spatial variations in plantlet growth in the culture vessel were reduced by increasing the forced ventilation rates and utilizing air distribution pipes.

In research by Ohyama and Kozai (1997), CO_2 concentrations in a test tube culture vessel (25 mm (1 inch) diameter, 120 mm (5 inches) height; commercially used in plant tissue culture) were measured under four levels of PPF during the photoperiod. It was shown that the CO_2 concentration inside the test tube containing a sweetpotato plantlet decreased linearly with increasing distances from the lid, indicating that the vertical distributions of CO_2 concentration and relative humidity were not uniform inside the test tube, which is relatively long and narrow in shape. Likewise, on a larger scale, nonuniform spatial distributions of CO_2 within a culture vessel can cause spatial variations of plantlet growth (Heo and Kozai, 1999). By using the present forced ventilation micro-propagation system with controllable ventilation and air distribution pipes, spatial variations in gaseous concentrations can be decreased significantly.

AIR CURRENT PATTERN INSIDE THE CULTURE VESSEL. The air current pattern inside the culture vessel in the FH treatment on day 6 is shown in Fig. 5. Upward and downward air currents were observed in the center and both sides of the culture vessel, respectively (Fig. 5A). The upward air current speed inside the culture vessel was approximately 2 $cm \cdot s^{-1}$ (0.8 inch/s). The air currents throughout the culture vessel with the air distribution pipes were relatively uniform, compared with the vessel without air distribution pipes (Fig. 5A and B). Increasing the forced ventilation rate during the culture period with the air distribution pipes in the culture vessel resulted in uniform spatial distributions of air current and CO_2 concentration near the air outlet which, subsequently, increased plantlet growth uniformity.

Table 2. Fresh weight, dry weight, and leaf area per sweetpotato plantlet on day 12. Means ±standard deviations are shown; FH = high forced ventilation, FM = medium forced ventilation, FL = low forced ventilation, and NE = extremely low natural ventilation.

Treatment code	Fresh wt (mg)[z]	Dry wt (mg)	Leaf area (cm^2)[z]
FH	511 ± 85	46.6 ± 8.0	9.1 ± 1.8
FM	563 ± 106	42.1 ± 8.6	9.0 ± 2.1
FL	559 ± 123	47.0 ± 12.9	7.9 ± 2.1
NE	415 ± 41	30.2 ± 0.8	5.3 ± 0.5

[z]28,350 mg = 1.0 oz.
[y]6.5 cm^2 = 1.0 $inch^2$.

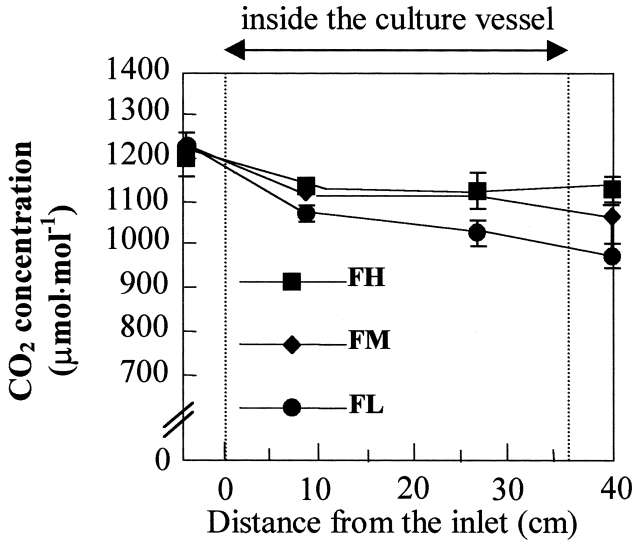

Fig. 4. Longitudinal CO_2 concentration inside the culture vessel on day 12. Vertical lines represent standard deviations (±2), n = 8; FH = high forced ventilation, FM = medium forced ventilation, FL = low forced ventilation, and NE = extremely low natural ventilation; 2.54 cm = 1.0 inch.

Conclusions

The growth of sweetpotato plug plantlets was improved in the forced ventilation micropropagation system with air distribution pipes at a CO_2 concentration of 1500 $\mu mol \cdot mol^{-1}$, a PPF of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and without sugar in the culture medium, as compared with plantlet growth in the standard, natural ventilation system.

Uniformity and promotion of plantlet growth was achieved by ar-

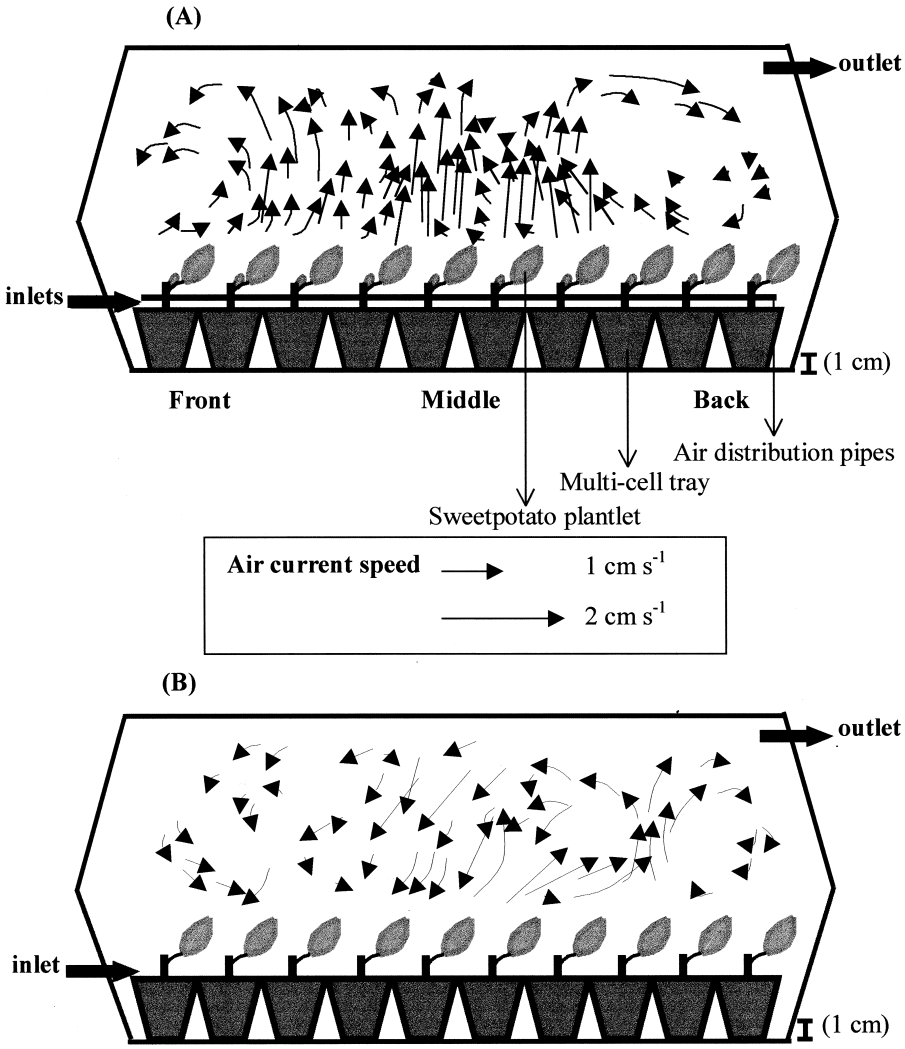

Fig. 5. Two dimensional patterns of air currents in the culture vessel with (A) and without (B) air distribution pipes under the forced ventilation. The forced ventilation rate was 13 mL·s^{-1} (0.8 inch^3/s) in both figures. The arrow length and its direction represent the air current speed and the direction of air current, respectively; 2.54 cm = 1.0 inch, 1 cm·s^{-1} = 0.4 inch/s.

ranging the air distribution pipes in the forced ventilated culture vessel to minimize spatial variations in the distributions of air current and CO_2 concentration. Commercialization of this system will require the development of an environmental control system to maximize photosynthetic (photoautotrophic) growth of plantlets by controlling CO_2 concentration, ventilation rate, and PPF; as well as relative humidity according to the growth stage of plantlets. Improvements of the system are currently underway.

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