Journal of Plant Biology, October 2007, 50(5) : 573-576

Biomass Production of *Anoectochilus formosanus* Hayata in a Bioreactor System

Yeo-Joong Yoon[1], Hosakatte Niranjana Murthy[1,2], Eun Joo Hahn[1], and Kee Yoeup Paek[1]*
[1]Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, South Korea
[2]Department of Botany, Karnatak University, Dharwad - 580 003, India

We investigated the factors that affect biomass production from *Anoectochilus formosanus* in a bioreactor system. Those factors included inoculum size, initial sucrose concentration, media supplements, photosynthetic photon flux density (PPFD), and culturing methods. An inoculum size of 8 g L^{-1} was most suitable for shoot proliferation; biomass accumulation was optimized when the medium was supplemented with 3% sucrose compared with sucrose-free media or those containing concentrations of 6% or 9%. This accumulation also was enhanced under a PPFD of 50 μmol m^{-2} s^{-1}. Likewise, the addition of coconut water (50 mL L^{-1}) plus activated charcoal (0.5 mg L^{-1}) to our Hyponex medium proved most beneficial. Comparative studies among three bioreactor systems – continuous immersion, raft (net), and temporary immersion (the ebb and flood system) – revealed that shoot proliferation and biomass accumulation were more efficient when culturing was performed under continuous immersion.

Keywords: Anoechtochilus formosanus, bioreactor culture, jewel orchid

Anoectochilus formosanus Hayata (Orchidaceae family) is an endangered orchid that is sparsely distributed in the Himalayan region of Indo-China, Taiwan, Vietnam, and Japan. It is popularly called the "Jewel Orchid" because of the network of colorful venation in its beautiful leaves. Although the flowers are not large, their white labella are quite prominent and toothed, such that a grouping of three or four flowering plants makes an attractive, unique addition to one's collection. *Anoectochilus* is also an expensive Chinese folk medicinal plant used to treat cancer, hypertension, diabetes mellitus, and nephritis in Taiwan (Liang et al., 1990). It is known as "King of Medicine" because of its diverse pharmacological effects, including anti-inflammation and heptoprotective activities (Lin et al., 1993), antioxidant activities (Lin et al., 2000; Wang et al., 2002), and antitumor and immunostimulating activities (Tseng et al., 2006). Gastrodin (4-(β-*D*-glucopyranosyloxy) benzyl alcohol), gastrodigenisn gastrodigenin (*p*-hydroxybenzyl alcohol), and kinsenoside (3-(R)-3-β-*D*-glucopyranosyloxybutanolide) are its most important bioactive components (Ito et al., 1993, Hsieh et al., 1997; Du et al., 2000), and can be obtained from whole-plant extracts (Du et al., 2003; Tseng et al., 2006).

Because this herb is precious in the Taiwanese market, its unrestricted harvesting from natural habitats has seriously reduced its populations (Shih et al., 2005). In addition, propagation by conventional methods is slow, and few tissue culture protocols have been developed for this important plant (Shiau et al., 2002; Ket et al., 2004). Therefore, to meet growing demand by the herbal and pharmaceutical industries, our major objective here was to develop a methodology for biomass production of *A. formosanus* Hayata using liquid media in airlift bioreactor cultures. We evaluated the parameters of inoculum density, initial sucrose concentration, photosynthetic photon flux density (PPFD), components of the culture medium, and specific culturing methods.

*Corresponding author; fax +82-43-272-5369
e-mail paekky@chungbuk.ac.kr

MATERIALS AND METHODS

Plant Material

Anoectochilus formosanus Hayata plantlets were grown *in vitro* on a 20-20-20 Hyponex (1 g L^{-1}; Kano, 1965) semi-solid medium (30 g L^{-1} sucrose, 2 mg L^{-1} benzyladenine, 1 g L^{-1} activated charcoal, and 2 g L^{-1} gelrite). Cultures were incubated at 25°C under a 16-h photoperiod, with a photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1}. The plantlets sub-cultured every four weeks and 1.0 to 1.5 cm-long shoots were used as explants.

Bioreactor Culture

Shoots (1.0 to 1.5 cm long, and containing a single node) were cultured in 5 L balloon-type bubble bioreactors with 3 L of a 20-20-20 Hyponex medium (enriched with 6.5N-4.5P-19K; 1 g L^{-1}) that was supplemented with 15 g L^{-1} sucrose, 2 g L^{-1} peptone, 50 mL L^{-1} coconut water, and 0.5 g L^{-1} activated charcoal (H1 medium). The pH was adjusted to 5.8 before autoclaving (30 min at 121°C and 1.2 kg cm^{-2} pressure). These bioreactor cultures were aerated at 0.1 vvm (air volume/culture volume per min), and were maintained at 25°C under lights (50 μmol m^{-2} s^{-1} PPFD, 16-h photoperiod). Five independent sets of experiments (inoculum density, sucrose, PPFD, media supplements, and bioreactor system) were conducted to optimize the production protocol. In the first set, cultures were established in an H1 medium at an initial inoculum density of 4, 8, 12, or 16 g L^{-1}. For our second set, cultures were treated in an H1 medium supplemented with 0, 3, 6, or 9% (w/v) sucrose. Here, an inoculum density of 8 g L^{-1} was used. In the third set, cultures were established by placing 8 g L^{-1} inoculum in an H1 medium supplemented with 3% sucrose, and then main-

taining them at 25°C either under darkness or while exposed to light (16-h photoperiod) from cool white fluorescent lamps at an intensity of 10 or 50 μmol m^{-2} s^{-1} PPFD. For the fourth set of experiments, we used the Hyponex medium (20N-20P-20K+6.5N-4.5P-19K; 1 g L^{-1} each) either alone or supplemented with 50 mL L^{-1} coconut water (CW), 0.5 g L^{-1} activated charcoal (AC), or a combination of CW and AC. These cultures were established with 8 g L^{-1} inoculum and were maintained at 25°C under lights (50 μmol m^{-2} s^{-1} PPFD, 16-h photoperiod). In the fifth set, we compared three types of bioreactor systems – continuous immersion, raft (net), and temporary immersion (ebb and flood), the last being programmed so that the explants were immersed in the medium for 30-min periods four times per day. In the raft method, the plants were held in place with a support net to avoid complete submersion in the liquid medium. All three types of cultures were established by using 8 g L^{-1} inoculum in an H1 medium supplemented with 3% sucrose, 50 ml L^{-1} CW, and 0.5 g L^{-1} AC. They were then maintained at 25°C under lights (50 μmol m^{-2} s^{-1} PPFD, 16-h photoperiod).

Growth Measurements

After eight weeks of culturing, all biomass was harvested and fresh weights were measured gravimetrically. Dry weights were then determined after the materials were oven-dried at 60°C for 2 d.

Experimental Design

A completely randomized design was applied, with three repetitions and three replicates. All data were subjected to Duncan's multiple range testes using a SAS program, and standard errors were calculated.

RESULTS AND DISCUSSION

Effect of Inoculum Density on Biomass Accumulation

In tests of inoculum density (Fig. 1), cultured shoots of *Anoectochilus formosanus* multiplied during the first four weeks, then developed roots, at their nodal regions from weeks 5 to 8. When an inoculum density of 4 g L^{-1} was used, flesh weight of the biomass was 237.1 g L^{-1}, with a corresponding dry weight of 23.5 g L^{-1}. Those values rose when a higher inoculum density, 8 g L^{-1}, was tested, resulting in optimum fresh (339.4 g L^{-1}) and dry (38.1 g L^{-1}) weights. These increments represented a 42-fold increase when compared with the initial fresh biomass. From studies with *Atropa*, Kanokwaree and Doran (1997) have also concluded that inoculum density is a critical factor influencing the final accumulation of biomass. Similar effects have been demonstrated during adventitious roots development in *Echinacea angustifolia* (Wu et al., 2006) and cell growth of *Gymnema sylvestre* (Lee et al., 2006).

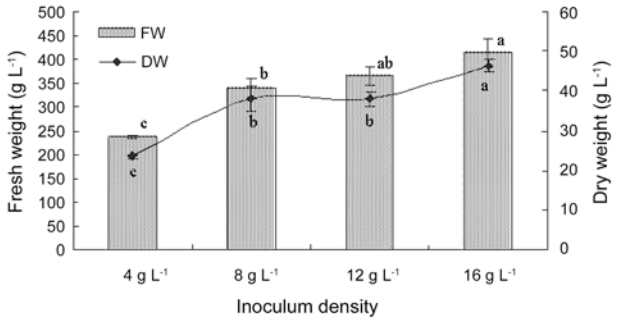

Figure 1. Effect of inoculum density on biomass accumulation by *A. formosanus* plantlets cultivated in bioreactor system. Mean values appear with SE (n=9); those marked with the same letters within the same parameter are not significantly different at *P* 0.05 (DMRT).

Effect of Sucrose Concentration on Biomass Accumulation

Sucrose is an important carbon and energy source for plant cell and tissue culture. Its initial concentration can affect growth and biomass accumulation (Desjardins et al., 1995). However, higher amounts can retard the development of cultured cells (Wu et al., 2006) by causing a cessation of the cell cycle when nutrients are limited (Gould et al., 1981). Here, cultures supplemented with 3% (w/v) sucrose were associated with significantly greater biomass accumulations (237.6 g L^{-1} and 27.2 g L^{-1} fresh and dry weights, respectively) compared with performance in the sucrose-free medium. In contrast, higher concentrations [6 or 9% (w/v)] were linked to reduced biomass production (Fig. 2).

Effect of PPFD on Biomass Accumulation

Culturing with illumination promoted better biomass

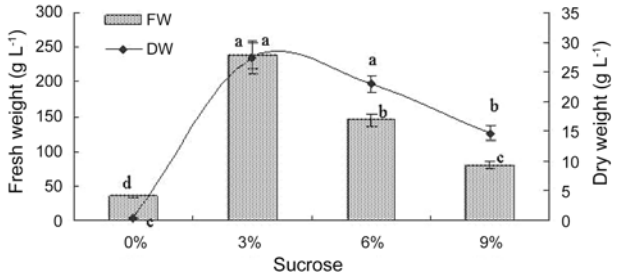

Figure 2. Effect of sucrose concentration on biomass accumulation by *A. formosanus* plantlets cultivated in bioreactor system. Mean values appear with SE (n=9); those marked with the same letters within the same parameter are not significantly different at *P* 0.05 (DMRT).

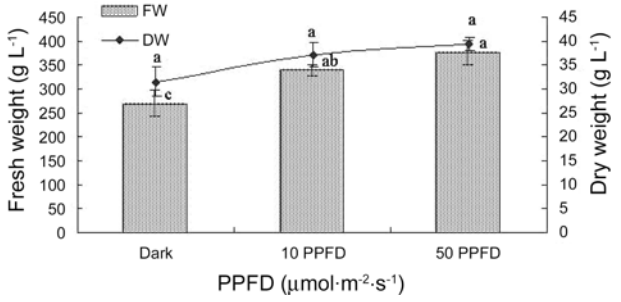

Figure 3. Effect of darkness and light on biomass accumulation by *A. formosanus* plantlets cultivated in bioreactor system. Mean values appear with SE (n=9); those marked with the same letters within the same parameter are not significantly different at *P* 0.05 (DMRT).

accumulations than when explants were treated under darkness (Fig. 3). The particular level of PPFD also was a factor. Here, a value of 50 μmol m^{-2} s^{-1} resulted in optimum shoot proliferation and biomass weights of 376.5 g L^{-1} (fresh) and 39.3 g L^{-1} (dry). Our observations are consistent with those of Escalona et al. (2003), who found that the photosynthetic rate responsible for higher accumulations in a temporary immersion bioreactor was maximum at high PPFD.

Effect of Media Supplements on Biomass Accumulation

The Hyponex medium is simple composition of nitrogen, phosphorous, and potassium, and is widely used for *in vitro* seed germination and propagation of orchids (Park et al., 2000). We tested four variations of this media type: 1) 20-20-20 Hyponex alone, 2) Hyponex supplemented with 50 ml L^{-1} coconut water (CW), 3) Hyponex plus 0.5 g L^{-1} activated charcoal (AC), Hyponex supplemented with 50 ml L^{-1} CW and 0.5 g L^{-1} AC. When just the standard mix was used, shoots proliferated and developed into plantlets within eight weeks, resulting in accumulations of 453.0 g L^{-1} fresh and 32.3 g L^{-1} dry weight (Fig. 4). No such benefit was achieved on the Hyponex medium supplemented with 0.5 g L^{-1} activated charcoal. However, fresh and dry biomass (759.7 g L^{-1} and 58.4 g L^{-1}) increased significantly when both coconut water and activated charcoal were added. Coconut water contains a wide spectrum of growth factors that can enhance the development of cultured cells and tissues (Shantz and Steward, 1952), and has been successfully used in orchid production (Murthy and Pyati, 2001; Payti et al., 2002). Moreover, activated charcoal may exert a positive influence by aborting various inhibitory substances, e.g., polyphenols that form on the medium (Fridborg and Eriksson, 1975).

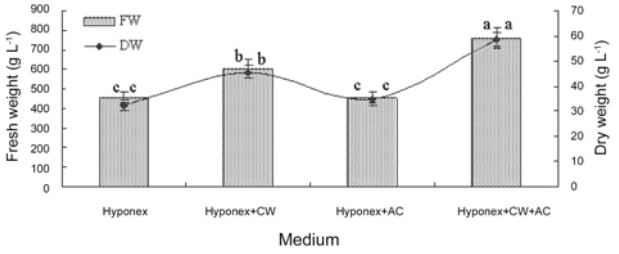

Figure 4. Effect of Hyponex medium and supplements on biomass accumulation by *A. formosanus* plantlets cultivated in bioreactor system. Mean values appear with SE (n=9); those marked with the same letters within the same parameter are not significantly different at *P* 0.05 (DMRT).

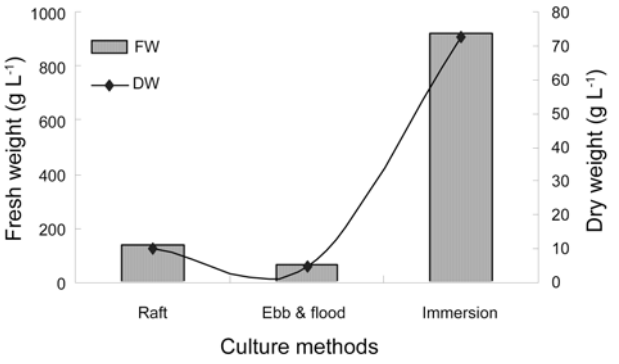

Figure 5. Effect of bioreactor system on biomass accumulation by cultured *A. formosanus* plantlets. Mean values appear with SE (n=9).

Effect of Culture Systems on Biomass Accumulation

Three types of bioreactor systems were investigated here: continuous immersion, raft culturing, and temporary immersion (ebb and flood). We found that neither ebb and flood no rafting was suitable (Fig. 5). Plantlets grew best under continuous immersion, as indicated by the highest biomass accumulations (919.2 g L^{-1} fresh biomass and 72.5 g L^{-1} dry biomass). Similar reports of success with that particular system have come studies of bulblet/shoot proliferation and biomass production in *Allium sativum* (Kim et al., 2004) and *Spathiphyllum cannifolium* (Dewir et al., 2006).

Our results demonstrate that optimizing all factors in the culturing protocol lead to a rapid, efficient rate of proliferation and maximum biomass accumulation during large-scale propagation of this important medicinal orchid. The biomass developed in a bioreactor system can then be used as raw material by the pharmaceutical and herbal industries because it has previously been demonstrated that tissue-cultured plants of *Anoectochilus formosanus* possess high amounts of active ingredients (Kinsenoside) (Do et al., 2001, 2003; Shih et al., 2005; Wu et al., 2007).

ACKNOWLEDGEMENTS

This work is financially supported partially by the Ministry of Education and Human Resources Development; the Ministry of Commerce, Industry, and Energy; the Ministry of Labor, and the Korean Science and Engineering Foundation, Government of Korea.

Received July 20, 2007; accepted August 21, 2007.

LITERATURE CITED

Desjardins Y, Hdider C, de Riek J (1995) Carbon nutrition *in vitro*. Regulation and manipulation of carbon assimilation in micropropagated systems. *In* Aitken-Christie J, Kozai T, Smith MAL, eds, Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Dordrecht, pp 441-472

Dewir YH, Chakrabarty D, Hahn EJ, Paek KY (2006) A simple method for mass propagation of *Spathiphyllum cannifolium* using an airlift bioreactor. In Vitro Cell Dev Biol Plant 42: 291-297

Du XM, Sub NY, Irino N, Shoyama Y (2000) Glycosidic constituents from *in vitro Anoectochilus formosanus*. Chem Pharm Bull 48: 1803-1804

Du XM, Sun NY, Tamura T, Mohri A, Sugiura M, Yoshizawa T, Irino N, Hayashi J, Shoyama Y (2001) Higher yielding isolation of Kinsenoside in *Anoectochilus* and its anti-hyperliposis effect. Biol Pharm Bull 24: 65-69

Du XM, Sun NY, Hayashi J, Chen Y, Sugiura M, Shoyama Y (2003) Hepatoprotective and anthihyperliposis activities in *in vitro* cultured *Anoectochilus formosanus*. Phytother Res 17: 30-33

Escalona M, Samson G, Bogroto C, Desjardins Y (2003) Physiology of effects of temporary immersion bioreactors on micropropa-

gated pineapple plantlets. In Vitro Cell Dev Biol Plant 39: 651-656

Fridborg G, Eriksson T (1975) Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol Plant 34: 306-308

Gould AR, Everett NP, Wang TL, Street HE (1981) Studies on the control of cell cycle in cultured plant cells. I. Effect of nutrient limitation and nutrient starvation. Protoplasma 106: 1-13

Hsieh MT, Wu CR, Chen CF (1997) Gastrodin and *p*-hydroxybenzyl alcohol facilitate memory consolidation and retrieval, but not acquisition, on the passive avoidance task in rats. J Ethnopharmacol 56: 45-54

Ito A, Kasai R, Yamasake K, Sugimoto H (1993) Aliphatic and aromatic glucosides from *Anoectochilus koshuensis*. Phytochemistry 33: 1133-1137

Kano K (1965) Studies on the media for orchid seed germination. Memories Fac Agric Kagawa Univ 20: 1-68

Kanokwaree K, Doran PM (1997) Effect of inoculum size on growth of *Atropa belladonna* hairy roots in shake flasks. J Ferment Bioengr 84: 378-381

Ket NV, Hahn EJ, Park SY, Chakrabarthy D, Paek KY (2004) Micropropagation of an endangered orchid *Anoectochilus formosanus*. Biol Plant 46: 339-344

Kim EK, Hahn EJ, Murthy HN, Paek KY (2004) Enhanced shoot and bulblet proliferation of garlic (*Allium sativum* L.) in bioreactor systems. J Hort Sci Biotech 79: 818-822

Lee EJ, Mobin M, Hahn EJ, Paek KY (2006) Effects of sucrose, inoculum density, auxins and aeration volume on cell growth of *Gymnema sylvestre*. J Plant Biol 49: 427-431

Liang WL, Chen RC, Chiang YJ, Su CH, Yang LL, Yen KL (1990) Study of *Anoectochilus* species. I. Study on the physiological activities of Jin-Sian-Lian. Formosan Sci 43: 47-58

Lin CC, Huang PC, Lin JM (2000) Antioxidant and hepatoprotective effects of *Anoectochilus formosanus* and *Gynostemma pentaphyllum*. Amer J Clin Med 28: 87-96

Lin JM, Lin CC, Chiu HF, Yang JJ, Lee SG (1993) Evaluation of the anti-inflammatory and liverprotective effects of *Anoectochilus formosanus*, *Ganderma lucidum* and *Gynostemma pentaphyllum*. Amer J Clin Med 11: 59-69

Murthy HN, Pyati AN (2001) Micropropagation of *Aerides maculosum* Lindl. (Orchidaceae). In Vitro Cell Dev Biol Plant 37: 223-226

Park SY, Murthy HN, Paek KY (2000) *In vitro* seed germination of *Calanthe sieboldi*, an endangered orchid species. J Plant Biol 43: 158-161

Pyati AN, Murthy HN, Hahn EJ, Paek KY (2002) *In vitro* propagation of *Dendrobium macrostachyum* Lindl. - A threatened orchid. Indian J Exp Biol 40: 620-623

Shantz EM, Steward FC (1952) Coconut milk factor: The growth promoting substance in coconut milk. J Amer Chem Soc 74: 6133-6135

Shiau YJ, Sagare AP, Chen UC, Yang SR, Tsay HS (2002) Conservation of *Anoectochilus formosanus* Hayata by artificial cross pollination and *in vitro* culture of seeds. Bot Bull Acad Sin 43: 123-130

Shih CC, Wu YW, Lin WC (2005) Aqueous extract of *Anoectochilus formosanus* attenuate hepatic fibrosis induced by carbon tetrachloride in rats. Phytomedicine 12: 453-460

Tseng CC, Shang HF, Wang LF, Su B, Hsu CC, Kao HY, Cheng KT (2006) Antitumor and immunostimulating effects of *Anoectochilus formosanus* Hayata. Phytomedicine 13: 366-370

Wang SY, Kuo YH, Chang HN, Kang PL, Tsay HS, Lin KF, Yang NS, Shyur LF (2002) Profiling and characterization of antioxidant activities in *Anoectochilus formosanus* Hayata. J Agric Food Chem 50: 1859-1865

Wu CH, Dewir YS, Hahn EJ, Paek KY (2006) Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. J Plant Biol 49: 193-199

Wu JB, Lin WL, Hsich CC, Ho HY, Tsay HS, Lin WC (2007) The hepatoprotective activity of kinsenoside from *Anoectochilus formosanus*. Phytother Res 21: 58-61