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Large-scale Culture of Plant Cell and Tissue by Bioreactor System

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Abstract

Large-scale cultures of plant cell, tissue, and organ have been achieved by using BTBB. When different sized BTBBs (5 L, 20 L, 100 L, 300 L, and 500 L) were tested for the culture of yew cells (*Taxus cuspidata* Sieb. et Zucc.), cell growth increment reached to 94.5% in SCV after 24 days of culture with 30% of inoculation cell density. However, there were some variations in the production of taxol and its derivatives among the BTBBs of different size. Approximate 4 mg/l of taxol and 84 mg/l of total taxanes were obtained by using a 500L BTBB after 6 weeks of culture.

With a 20L BTBB, about 20,000 cuttings of virus-free potatoes (cv. Dejima) could be obtained by inoculating 128 explants and maintaining 8 weeks under 16 hr light illumination. The frequency of ex vitro rooting of the cuttings revealed as more than 99% under 30% shade.

By incorporating two-stage culture process consisting of multiple bulblet formation in solid medium and bulblet development in liquid medium, mass propagation of lily through bioreactor seemed to be possible. In the case of 'Marcopolo', the growth of mini-bulblets in BTBB was nearly 10 folds faster than that of the solid medium.

Time course study revealed that maximum MAR yield of ginseng (*Panax ginseng* C. A. Meyer) in a 5 L and 20 L BTBB after 8 weeks of culture was 500 g and 2.2 kg, respectively. By cutting the MAR once and/or twice during the culture, the yield of root biomass could be increased more than 50% in fresh weight at the time of harvest. With initial inoculum of 500 g of sliced MAR in a 500 L BTBB, 74.8 kg of adventitious root mass was obtained after 8 weeks of culture. The average content of total ginseng saponin obtained from small-scale and/or pilot-scale BTBBs was approximately 1% per gram dry weight. Based on our results, we suggest that large-scale cultures of plant cell, tissue, and organ using BTBB system should be quite a feasible approach when compared with conventional method of tissue culture.

Abbreviations: BTBB, balloon type bubble bioreactor; SCV, sedimedted cell volume; MAR, multiple adventitious root

Introduction

Plants provided useful sources of a vast array of natural products used as foods, drugs, pesticides, flavorings, and fragrances etc. [3]. These plants originated biochemical and/or biomass have traditionally been obtained by harvesting field grown plants, but most of useful substrates were produced by chemical synthesis which was often more reliable, consistent, and cost reducing method [16]. Plant cell and tissue culture technology was suggested as an attractive approach to supply plant-derived sources especially when the plant itself was difficult to cultivate, has a low secondary metabolite yield, or chemical synthesis has not been achieved due to complicated structure. By considering economic feasibility, large-scale

cultures of plant cell and tissue through bioreactor system were proposed, previously [2, 6, 25]. Nevertheless, there are few reports on the success of industrial-scale culture of plant cell, tissue, and organs via bioreactor because of the lack of knowledge [15, 26]. To adapt the technology used for the fermentation of microbials into the culturing of plant material, STR was introduced for scale-up process, recently [8]. Although STR have many merits to culture of plant materials, problem related to shear force and contamination during long period of culture are still existed [20, 21, 27]. Scant attention has been given to the air-lift bioreactor because of some difficulties to scale-up due to their huge size in appearance [14]. Cultures of yew tree cell, potato seedling, lily bulblet, and MAR of ginseng were tested in this study to investigate their potencies for mass production in bioreactor. In this study, novel type BTBB was also incorporated to verify the effectiveness for scale-up of plant cell, tissue, and organ culture with different genotype.

Materials and Methods

Plant materials

Collected immature seeds of yew trees were disinfected by soaking for 4 hr in running tap water; immersed in 70% ethanol for 1 min; immersed in 2% sodium hypochloride having two drops of Tween-80 under vacuum condition for 45 min; followed by five rinses in sterilized distilled water(SDW). Initial culture was conducted by inoculating dissected embryos from immature seeds. Culture medium was prepared by incorporating mB 5 [11] basal salt supplemented with 45.24 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 500 mg/l casein hydrolysate, 250 mg/l myo-inositol, 0.5 mg/l gibberellic acid (GA_3), 2×MS vitamin [22], 0.15% (w/v) gelrite, and 3% (w/v) sucrose. Once calli were induced and proliferated, only the surface of the callus showing rapid growing behavior was isolated and subcultured at the minimum size of 0.5 cm in their diameter. Subculture was routinely conducted at 4 weeks interval. Cell suspension culture was established by inoculating about 5 to 6 g fresh weight of callus originated from selected cell lines into 250 ml Erlenmeyer flask containing 40 ml of previously described mB5 liquid medium having 9.05 μM 2, 4-D and agitated at 110 rpm with gyratory shaker installed in a thermostatically controlled room at 26℃ under complete dark condition. After 6 times of serial subcultures, cultures in exponential phase (1 week after inoculation) were filtered through the double fold sieve (pore size in 0.1×0.1 cm) and collected fine cells were used as source for the bioreactor culture.

Virus-free *in vitro* seedlings were kindly provided from the Koryunggi Experimental Station in Rural Development Administration (RDA). After multiplicating the shoots in MS medium containing 0.89 μM 6-benzyladenine (BA), each shoot was elongated on the same medium without plant growth regulator (PGR) until the height and diameter of the shoot reached to 8 cm, and 0.4 cm, respectively in hexahedron glass vessel (8.5 × 8.5 × 14 cm). Before inoculating just mentioned above shoot cultures into the BTBB, apical parts, some expanded large leaves, and root system were removed. Stem explants having small leaves were divided into 2 cm length and transferred into the BTBB, aseptically.

To proliferate multiple mini-bulblets from *in vitro* grown lily bulblet, bulb-sacle was isolated, divided into 3 pieces by vertical slicing, and cultured onto MS medium supplemented with 4.44 μM BA, 1.61 μM α-naphthaleneacetic acid (NAA), 0.18% gelite, and 3% (w/v) sucrose. When the diameter of newly induced mini-bulblet reached to 3 mm, the bulblets were harvested, checked contamination, then used as the source for bioreactor culture.

Six-year-old ginseng roots were collected and surface-sterilized with 70% ethanol for 30 sec; immersed in 2% sodium hypochlorite containing two drops of Tween-20 for 3 min; and finally rinsed five times with SDW. Inside of the surface sterilized ginseng roots were sliced into small pieces (0.16 cm^3) and cultured onto plastic Petri dish (12 × 1.5 cm) containing 20 ml of solid SH basal medium [24] supplemented with 4.9 μM 3-indole butryic acid (IBA), 3% sucrose, and 0.15% gelrite. When callus was induced, only callus positioned on the surface of the explants was subcultured at every 2 week on the same medium described above. Adventitious roots from the callus were obtained by culturing newly proloferated callus onto the same medium having 24.60 μM IBA. To induce MARs from *in vitro* originated roots, each roots were isolated from callus tissue then prepared into 2 cm in length before to culture onto plastic Petri dish containing 20 ml of solid MS basal medium supplement with 24.60 μM IBA. Cultures were maintained for more than two months in liquid medium by shaking before using as

a source for this scale-up study.

Bioreactor culture

To culture of yew tree cells, small-scale (20 L) and/or pilot-scale (100 L to 500 L) BTBBs were used. The initial cell density was adjusted to 30% (v/v) and culutres was maintained at 26℃ under completely dark condition.

For the culture of potato shoots, 128 piece of stem cuttings were inoculated onto a 20 L BTBB having stainless steel sieve (pore size in 0.1 × 0.1 mm). To generate ebb and flow circulation of medium, timer and solenoid valve were installed. Dual reservoir tanks were attached to the BTBB to supply shoot multiplication medium for the first two weeks and to switch shoot elongation medium for further 4 weeks. Because the 20 L BTBB was made by glass, light was illuminated by installing round flourescence lamp upside to the bioreactor.

Mini-bulblets harvested from solid medium were cultured onto a 1 L sized conical flask for 7 days to investigate the evidence of contamination. Around 100 mini-bulblets were inoculated onto a 20 L BTBB having 15 L of PGR free MS medium containing 9% (w/v) sucrose and 0.1% (w/v) activated charcol.

To investigate the pattern of ginseng root growth in a small-scale BTBB, roots derived from suspension culture were prepared by cutting the source using top motor driven blade, and the inoculum was adjusted to 240 g fresh weight into a 20 L BTBB. To investigate the effect of root cutting, the increment of biomass before and after cutting were evaluated. To establish pilot-scale culture, cultured MARs were cut, placed on a reservoir and then took for 7 days in order to check contamination before transferring to 100 L, 300 L, and 500 L BTBBs. Inoculum for each pilot-scale BTBB was 1% (w/v) at fresh weight base.

To give constant temperature, a small-scale bioreactor (20 L) which made by glass was installed in a thermostatically controlled room. Whereas, the temperature of cultures in pilot-scale bioreactors (100 L to 500 L) were controlled by circulating tempered water through outside jacket. In addition, the temperature of input air was also adjusted to 20 to 26℃ by air dryer. Sparging air volume was adjusted with air flow meter (RMA 14, Dwyer Co., USA) to supply constant flow rate of 0.02 to 0.05 vvm.

Field test

When the height of potato shoots in a 20 L BTBB reached about 18 cm, they were sliced with one or two nodes, then transplanted onto pots filled with vermiculite on the greenhouse. During the *ex vitro* rooting period, 30% shade and more than 90% relative humidity were applied. After 4 weeks later, each plantlet was eventually transferred to the field to investigate further growth.
Lily bulblets, which diameter range was from 4 cm to 6 cm, were harvested from the BTBB, then washed 3 times with tap water and finally transplanted onto the pot containing artificial soil mix.

Analysis of extract

To investigate the content of taxanes from yew tree cell cultures, cells and medium were separated by centrifugation at 11,000 rpm for 1 min followed by filtration. Collected medium was fractionated using Sep-pak C_{18} cartridges (Millipore Corp., Milford Massachusetts, USA), treated in advance with 5 ml of water followed by the same volume of acetonitrile, and diluted with high performance liquid chromatography (HPLC) grade methanol. To prepare cell extracts, cells were immersed in the same volume of methanol, sonicated for 45 min, and filtered through Whatman filter paper (No. 1). The extracts from cells and medium were mixed together and final volume was adjusted to 1 ml with methanol before injection into the HPLC. Taxane analysis was done by HPLC method described previously [12] using a Curosil G column (Phenomenex 6μ, 3.2×250 mm) with slight modification. Taxanes were analyzed under isocratic condition that consisted of acetonitrile and distilled water in 40:60 ratio as mobile phase. The flow rate was set to 0.6 ml/min. Effluents of taxanes were monitored at 229 nm by photo diode array (PDA) detector.

To analysis of ginseng saponin, dried matter of MAR (1 g) were extracted with 60% methanol (40 ml) at 100℃ for 3 hr and filtered through filter paper (Whatman No. 1) under vacuum condition. The extract was evaporated to dryness and dissolved in 10 ml of HPLC grade water. The water-soluble extract was passed slowly through a Seppak C_{18} cartridge (Waters, USA), then eluted with 10 ml of methanol. The saponin fraction was analyzed by using HPLC system (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millenium 2010 chromatography manager) on a Altec Platinum C_{18} column (1.5 μ, 33 × 7 mm,) with water and a-

cetonitrile. The rate of water and acetonitrile for first 10 min and late 15 min were 75:25 and 63:37, respectively. Flow rate of the mobile phase was 1.2 ml/min and monitoring of ginsenoside was 203nm.

Results and Discussion

In order to increase the chance of obtaining high taxol producing cell lines, seeds from different trees were collected and used as the source for culture initiation. Possibly due to either the diverse genetic background or different physiological states of the seeds, cell cultures originated from embryos revealed significant variation in their growth behavior as well as taxane contents [18, 23, 28]. The excised embryos from immature seeds harvested in late September appeared to be the best source for callogenesis with almost 100% of response and the greatest cell proliferating capacity. Whereas, those from mature seeds resulted in lower than 50% of callogenesis (data not shown). BTBB gave remarkably the highest cell growth increment. Total SCV in 20 L BTBB reached 94.5% after 24 days of culture with 30% of inoculation cell density. The doubling time was approximately 12 days based on SCV. This was much faster than that observed by previous report [7] which referring the doubling time in cell suspension of *T. cuspidata* was approximately 17 days and 20.6 days based on fresh weight and dry weight, respectively. When the medium was sterilized at 121℃ for 45 min, one sixth of sucrose was hydrolyzed into glucose and fructose. Sucrose was absorbed and/or totally hydrolyzed within 6 days of culture. On the other hand, the levels of glucose and fructose in the medium increased from day 3 to 6 then decreased therefrom. The pattern of carbohydrate consumption and/or accumulation in medium at early stage of culture was comparable to those reported previously [7]. The increment of glucose and fructose level seemed due to the secretion of sucrose invertase from actively growing cells and by hydrolysis of the disccharide [5]. During the early exponential phase, glucose and fructose consumed simultaneously in different degrees. Glucose had almost disappeared by the day 12. This can be explained by the fact that glucose is preferred to fructose as a carbon source in this cell line [7]. When both glucose and fructose in culture medium were completely exhausted at the day 21, growth phase switched from exponential to deaccelerated exponential and/or early stationary phase. The result suggests that the best time for the replacement of fresh medium in batch culture and/or medium addition in fed batch culture using BTBB are 12 to 15 days from culture initiation just before sugar starvation. When a 500 L size BTBB was used for batch culture of *Taxus cuspidata*, almost the same pattern of cell growth was observed compared to that in 20 L BTBB. In pilot-scale culture of yew tree cells, it was difficult to supply fixed amount of air due to the large volume of cells in the bioreactor. To overcome the difficulties, air flow was adjusted by monitoring cell circulation. The cell viability of the 500 L grown cultures was more than 70%. Taxol could usually be detected within 12 days of culture. The average yield of taxol at the time of harvest was 3 to 5 mg/l at 27 days of culture. Although this productivity level only slightly exceeds that reported with *T. baccata* [7] and *T. cuspidata*[29] based on a month cycle, this is the first report describing the production of taxol in a pilot-scale culture of yew cells. Taxane production routinely started from 5 days after inoculation and increased in a linear fashion until day 30, then decreased thereafter. Interestingly, the yield of total taxanes after 42 days of culture reached 84 mg/l which corresponds to a maximum volumetric productivity of 2. 74 mg/l per day. Although more detailed studies are needed to convert taxanes into taxol in *vitro*, high productivity of total taxanes in this study indicates the possibility of using a BTBB system for the large-scale production of taxol (Figure 1a-1c).

Multiplication of potato seedlings *in vitro* was conducted by inoculating stem segments (1 cm in length) into vessels containing solid MS medium as demonstrated previously [1]. After sufficient shoot cultures were obtained, each shoot was subcultured onto hexahedron glass vessel (8 × 8 × 14 cm) having micro-filter. After 4 weeks, shoot height and stem diameter reached to 12 cm and 0.4 cm, respectively. Furthermore, the shoot cultures represented healthy expanded leaves. The reason for the rapid growth performance seemed partly due to high gas exchange capacity of the vessel [13]. Because the number of shoot segments inoculated into the BTBB significantly affected shoot growth, yield of micro-tuber and/or the number of shoot cuttings at the time of harvest, preliminary experiment was conducted to verify suitable density of inoculum (data not shown). With initial culture of 128 stem segments, average 680 micro-tubers (average diameter less than 1 cm) were obtained by placing a BTBB for 4 weeks at 26℃ under 16 hr light illumination and

Figure 1. The use of balloon type bubble bioreactor (BTBB) to culture plant cell, tissue, and organ. a. Actively growing cells of yew tree (Taxus cuspidata Sieb. et Zucc.). b. 20 L sized BTBB used for yew tree cell culture. c. Pilot-scale bioreactor systems. d. Potato seedlings growing in a 20 L BTBB. e. Micro-tuber formation in a 20 L BTBB. f. Establishment of pot plants of potato by ex vitro rooting. g. Mini-bulblets at the time of inoculation in a 20 L BTBB. h. After 2 weeks of culture. i. After 4 weeks of culture. j. At the time of harvest of ginseng roots in a 500 L BTBB. k. Cutting of multiple adventitious roots during the culture with blade. l. Harvested ginseng roots.

another 4 weeks at 18℃ under complete dark condition. Subjecting potato shoot segments to transient immersion in a BTBB, 12 to 24 cycle/day with immersion period of 30 min was found to be adequate. Shoot cuttings for *ex vitro* rooting were conducted by harvesting shoot cultures reached to 18 cm in their height and slicing the stem. Total cuttings obtained from a 20 L BTBB were around 20, 000 (Figure 1d-1f). Acclimatization was performed by transplanting the shoot cuttings into pot containing perlites for 2 weeks under 30% shade. At that time, relative humidity was maintained up to 90% in greenhouse. When root system was formed, the seedlings were re-planted onto the plastic pot containing artificial soils and liquid fertilizer (20-10-20) was applied once per 2 weeks. As shown in figure 1-e, the seedlings showed normal growth pattern compared with that from seed potato.

Figure 1-g to 1-i illustrates the growth and development of bulb-scale originated micro-bulblet onto a 20 L BTBB containing MS medium supplemented with 9% (w/v) sucrose. The immersion of bulblets in a BTBB was possible by generating ebb and flow of medium circulation by solenoid valve and timer. Although the bulblets continuously swelled and their diameter reached to 5 cm after 4 months of culture in the BTBB, multiplication of new bulblets was also developed spontaneously (see Figure 1-i). In the case of bulblets obtained by culturing in a BTBB, 98% success in rooting and sprouting were observed by transplanting in pots containing artificial soil. The procedure used here was much simpler than general tissue culture methods because frequent subculturing steps were eliminated.

Growth and harvest of MAR cultures on pilot-scale bioreactor is shown in figure 1-j to 1-l. Maximum biomass of 500 g and 2.2 kg fresh weight in a 5 and 20 L bioreactor were obtained after 42 days from inoculation. The equation obtained by culturing MAR in pilot-scale bioreactor for 8 weeks was $y = 569.55e^{0.073x}$ ($R^2 = 0.974$). The yield of ginseng saponin was increased until the MAR growth reached to the end of the exponential growth phase and decreased thereafter. When the cultured MARs were cut with blade (Figure 1-k) once (at 4 week) and/or twice (at 4 week and 6 week) during the culture, the yield of biomass sharply increased. In the case of once and/or twice cuttings, the fresh weight of MARs reached to almost 2.8 kg after 8 weeks of culture in 20 L BTBB. The pattern of MAR growth and saponin increment seemed to be similar in different size (100 L, 300 L, and 500 L) of BTBBs tested. Although explant cutting for the separation of initial cultures at the time of inoculation had been routinely conducted in many publications [17, 19, 30], the effect of root cutting during the culture for the increment of biomass were not investigated before. At the time of harvest, root system routinely tangled together and formed ball-like structure without any cutting treatment. When the tangled MARs were sliced vertically, inside of the mass revealed as brown color. In addition, the ginsenoside content of the brown parts of roots highly decreased compared with that of actively growing roots positioned surface of the mass. By operating top driven motor having sharp blade, tangled MARs could be easily cut into 2 to 3 cm size. When root cutting treatment was applied in small-scale to pilot-scale BTBBs, the productivity of root biomass at harvest was increased around 50% compared with that of control. Previous studies revealed that growth rate of some types of ginseng cells and tissues were various, for example, 2 to 10 folds at 21 to 28 days of culture in cell suspension [2, 4], 1.5 to 8.2 folds at 20 to 30 days of cultu

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