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# Aeration Volume and Photosynthetic Photon Flux Affect Cell Growth and Secondary Metabolite Contents in Bioreactor Cultures of *Morinda citrifolia*

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**To improve their growth and secondary metabolite production, we cultured *Morinda citrifolia* leaf cells for 3 weeks in bioreactors with different aeration volumes (0.05, 0.1, 0.2, or 0.3 vvm; or 0.05/0.1/0.2/0.3 vvm, as increased at 5-d interval), and photosynthetic photon fluxes (PPF; 0, 15, 30, or 45 μmoL $m^{-2}$ $s^{-1}$). Cell growth was greatest (15.6 g $L^{-1}$ dry weight) at 0.3 vvm whereas the accumulation of secondary metabolites (total anthraquinones, phenolics, and flavonoids) was maximized at 0.1 vvm. A PPF of 15 μmoL $m^{-2}$ $s^{-1}$ accelerated the accumulation of both cell biomass and metabolites. Dark-culturing suppressed cell growth, while a high PPF (45 μmoL $m^{-2}$ $s^{-1}$) inhibited metabolite biosynthesis. Further studies are required to understand the reason for differences in the effect of light on cell growth and secondary metabolite contents in *M. citrifolia* cell cultures.**

Keywords: aeration volume, anthraquinones, cell culture, flavonoids, *Morinda citrifolia*, phenolics, photosynthetic photon flux (PPF)

*Morinda citrifolia* L. plants have been used for over 2000 years as traditional food and folk medicine in Polynesia (Wang et al., 2002). This species consists of three varieties -- citrifolia, bracteata, and potteri -- with citrifolia, aka 'Noni', being the most famous (Nelson, 2001). It contains several types of bioactive compounds, e.g., terpenoids, alkaloids, anthraquinones, sitosterol, carotene, flavone, and glycosides. For medicinal purposes, plants require approx. 2 years to mature, and must grow in regions with high temperatures and humidity levels. Unfortunately, continuous harvesting in natural settings has caused the significant depletion of mother plants.

Current advances in plant biotechnology make cell and organ culturing a promising approach for producing useful secondary metabolites. However, practical application has been limited because most cultures have been optimized only on a small scale (i.e., in flasks). Therefore, to fulfill higher demand, cultures should be maintained in large-scale bioreactors designed for intensive production, with monitoring and control of such physical factors as aeration, temperature, gaseous composition, pH, and light (Paek et al., 2005). Aeration volume is one of the most important components affecting these bioreactor cultures because it is closely related to the agitation of explants and dissolved oxygen in the media, which regulate the accumulation of biomass and secondary metabolites (Shohael et al., 2005; Lee et al., 2006). Illumination (photoperiod and light quality) is another important ingredient in bioreactors (Ali et al., 2005; Wu et al., 2007). These factors must be optimized according to plant species and explant type (cell, tissue, or organ) to maximize the production of both biomass and desirable compounds.

In the case of *Morinda citrifolia*, Zenk et al. (1975) have shown that anthraquinone content in leaf cells is more than 10 times higher compared with differentiated roots. However, no details have been reported on the physical and chemical factors that affect cultures and secondary metabolite production in cells of that species. Here, we optimized the culture medium through experiments on salt strength, nitrogen source, sucrose concentration, and growth regulators (see also Saifullah, 2007). Based on those preliminary results, we then transferred the cells to bioreactors to investigate the effects of aeration volume and photosynthetic photon flux (PPF) on biomass and secondary metabolite contents.

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## MATERIALS AND METHODS

### Induction and Proliferation of Calli

Calli were induced from *in vitro* plantlets of *Morinda citrifolia*. Leaves from the apical buds were cut into 1×1 cm pieces, then inoculated in Petri dishes containing 25 mL of an MS solid medium (Murashige and Skoog, 1962) supplemented with 2.0 mg $L^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D), 1.0 mg $L^{-1}$ kinetin, 30 g $L^{-1}$ sucrose, and 2.3 g $L^{-1}$ gelrite. To induce calli, cultures were maintained for 4 weeks in the dark at 25±2°C. Afterward, those calli were transferred to 400-mL flasks containing 100 mL of an MS liquid medium supplemented with 3.0 mg $L^{-1}$ NAA, 0.1 mg $L^{-1}$ kinetin, and 30 g $L^{-1}$ sucrose. The calli were proliferated at 25±2°C under a 16-h photoperiod and a PPF of 20 μmoL $m^{-2}$ $s^{-1}$ (Saifullah, 2007).

### Bioreactor Cultures

*Effect of aeration volume*

*M. citrifolia* cells were cultured in 3-L balloon-type airlift bioreactors (Fig. 1), in which the aeration volume was set at 0.05, 0.1, 0.2, or 0.3 vvm, or at 0.05/0.1/0.2/0.3 vvm (gradually increased at 5-d intervals). Cell growth (fresh and dry weights, and % dry weight) was evaluated after 3 weeks, along with the contents of secondary metabolite contents (anthraquinones, phenolics, and flavonoids). Each experi-

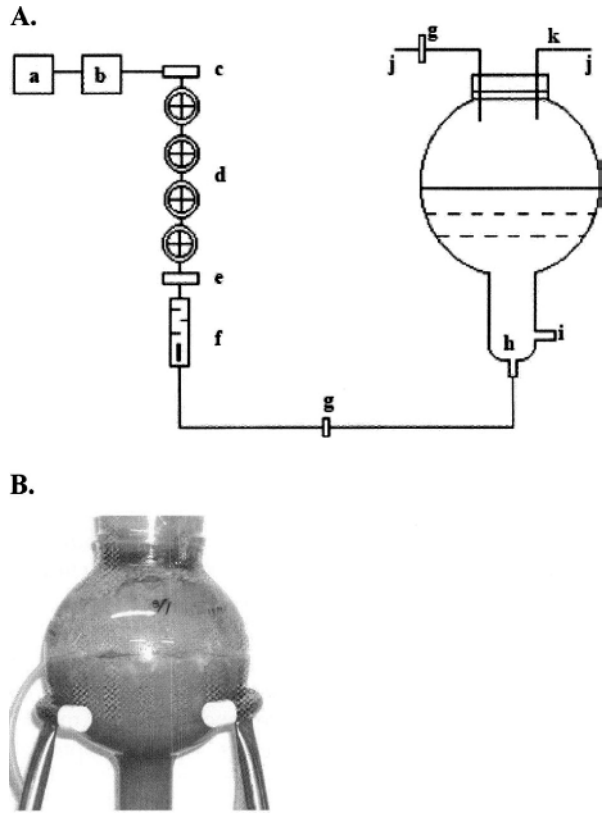


**Figure 1.** **A,** Schematic diagram of airlift bioreactor. a: air compressor, b: air reservoir, c: air cooling device, d: air filter system, e: air dryer, f: air flow meter, g: membrane filter, h: glass sparger, i: medium feeding port, j: vent, k: pre-filter. **B,** *Morinda citrifolia* cells cultured in bioreactor.

ment was repeated twice, and included three replications per treatment.

*Effect of PPF*

Cells were cultured in a growth chamber for 3 weeks in 3-L balloon-type airlift bioreactors under different PPF levels: 0, 15, 30, or 45 μmoL $m^{-2}$ $s^{-1}$. The aeration volume was adjusted to 0.3 vvm, a setting that had been determined to be optimal during the experiment described previously. Measurement parameters were the same as those above.

### Determination of Cell Growth Rate (GR)

The growth rate (GR) of cells after 3 weeks of culturing was calculated as:

GR = [{harvested dry weight (g) – inoculated dry weight (g)}/ inoculated dry weight (g)]/culture period (d).

### Determination of Total Anthraquinone Content

Anthraquinone was measured spectrophotometrically according to the method of Zenk et al. (1975). Briefly, 0.1 g samples of dried cells were extracted by heating in 15 mL of 80% ethanol at 80°C for 2 h, after which the ethanol was collected. This procedure was repeated until the final extractant was colorless. After the volume was made up to 50 mL, the ethanol fractions were pooled and filtered through 0.45 μm Whatman micro filters. The anthraquinone content was determined with a UV-visible spectrophotometer (UV-1650PC, Shimadzu, Japan) at 434 nm, using alizarin (Sigma, Germany) as a standard (Stalman et al., 2003).

### Determination of Total Phenolics Content

The same extracts were analyzed spectrophotometrically by following a modified version of the Folin-Ciocalteu colorimetric method (Shohael et al., 2006). Briefly, 100 μL of ethanolic extracts was combined with 2.5 mL of de-ionized water before 0.1 mL of (2N) Folin-Ciocalteu reagent (Sigma, Germany) was added. These were mixed well, then allowed to stand for 6 min before the addition of 0.5 mL of a 20% sodium carbonate solution. The color developed after 30 min at room temperature and absorbance was measured spectrophotometrically at 760 nm. Our measurements were compared to a standard curve for a prepared gallic acid solution, and the phenolics content was expressed as the equivalent mg of gallic acid (Sigma, Germany) per gram of dry cells (Wu et al., 2006).

### Determination of Total Flavonoid Content

Total flavonoid content was assessed according to the colorimetric method (Shohael et al., 2006), in which 0.25 mL of ethanolic plant extract or a catechin standard solution was mixed with 1.25 mL of distilled water, followed by the addition of 0.75 mL of a 5% sodium nitrite solution. After 6 min, 0.15 mL of a 10% aluminum chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 mL of 1M sodium hydroxide was added. This mixture was brought to 2.5 mL with distilled water and mixed well. Absorbance was measured immediately at 510 nm with a spectrophotometer, and the results were expressed as mg of catechin equivalent per gram of dry cells for the triplicate extracts.

## RESULTS AND DISCUSSION

Fresh and dry weights and the % dry weight of *Morinda citrifolia* leaf cells increased in proportion to aeration volume, showing maximal growth (15.6 g $L^{-1}$ DW) at 0.3 vvm (Table 1). A gradual increase also was found from 0.3 to 0.5 vvm (14.71 g $L^{-1}$ DW). We previously showed that a gradual rise in aeration volume is favorable to growth in bioreactors because the high inflows of air agitate cells, thereby elevating the concentration of dissolved oxygen in the culture

**Table 1.** Fresh and dry weights, % dry weight, and growth rate of *Morinda citrifolia* cells as affected by aeration volume after 3 weeks of bioreactor-culturing.

| Aeration volume (vvm) | Fresh weight (g $L^{-1}$) | Dry weight (g $L^{-1}$) | % dry weight | Growth rate (g DW $d^{-1}$) |
|---|---|---|---|---|
| 0.05 | 476.59±3.64[Z] | 11.78±0.61 | 2.47 | 0.77 |
| 0.1 | 504.79±10.28 | 13.53±0.40 | 2.68 | 0.90 |
| 0.2 | 527.44±7.84 | 13.85±0.90 | 2.62 | 0.92 |
| 0.3 | 569.40±12.37 | 15.60±1.50 | 2.74 | 1.04 |
| 0.05~0.3 | 560.54±10.24 | 14.71±0.74 | 2.62 | 0.98 |

[Z]Values are means and standard deviations for 3 replicates.

**Table 2.** Effect of aeration volume on secondary metabolite contents in *Morinda citrifolia* cells after 3 weeks of bioreactor-culturing.

| Aeration volume (vvm) | Anthraquinones | | Phenolics | | Flavonoids | |
|---|---|---|---|---|---|---|
| | Total (mg $g^{-1}$ DW) | Yield (mg $L^{-1}$) | Total (mg $g^{-1}$ DW) | Yield (mg $L^{-1}$) | Total (mg $g^{-1}$ DW) | Yield (mg $L^{-1}$) |
| 0.05 | 10.36±0.38[z] | 122.04 | 9.45±0.25 | 112.38 | 8.21±0.40 | 96.71 |
| 0.1 | 18.88±0.23 | 255.45 | 18.12±0.13 | 245.16 | 13.55±0.35 | 183.33 |
| 0.2 | 18.33±0.05 | 253.87 | 13.78±0.26 | 190.85 | 12.25±0.57 | 169.66 |
| 0.3 | 17.46±0.08 | 272.38 | 11.50±0.06 | 179.40 | 11.68±0.19 | 182.21 |
| 0.05~0.3 | 7.35±0.07 | 108.12 | 8.40±0.10 | 123.56 | 7.97±0.14 | 117.24 |

[z]Values are means and standard deviations for 3 replicates.

while accelerating cell growth, whereas maintaining a constant, high aeration volume throughout the culture period inhibits their growth due to sheer stress (Jeong et al., 2006; Lee et al., 2006). However, we found here that cell growth was not negatively affected by a high aeration volume (0.3 vvm), a level that resulted in the greatest growth rate (Table 1). The reason for this might be that leaf cell growth was faster at the beginning of the culture period (Days 2 to 3; Lee et al., 2006) compared with that of the adventitious roots (Days 5 to 7; Jeong et al., 2006; Wu et al., 2007). In *M. citrifolia*, therefore, a high aeration volume increases the supply of oxygen to the cultures and enhances cell growth without causing sheer stress.

The highest contents of total anthraquinones, phenolics, and flavonoids were obtained at an aeration volume of 0.1 vvm, with values declining as that volume rose. Gradual increases, from 0.05 to 0.3 vvm, negatively affected the accumulation of secondary metabolites, resulting in the lowest contents (Table 2). In general, a high aeration volume is beneficial in speeding up the transfer of oxygen into bioreactors, a process that improves both secondary metabolite accumulation and cell growth (Zhong et al., 1993). However, such a high volume is not always advantageous to the accumulation of compounds in plant cultures. Min et al. (2007) have reported higher biomasses from *Scopolia parviflora* when cultured in a high aeration volume (0.4 vvm), but increased alkaloid contents at a lower volume (0.1 vvm). Those results suggest that aeration volume should be adjusted based on the explant source selected, as well as according to the cultural stage for either biomass production or the storage of secondary metabolites.

In our PPF experiments, the accumulation of cell biomass (Table 3) was greatest at 15 μmoL $m^{-2}$ $s^{-1}$ (486.24±4.03 g $L^{-1}$ FW; 13.06±0.68 g $L^{-1}$ DW). In contrast, culturing under darkness severely suppressed growth, leading to the lowest values for fresh and dry weights (160.43±8.82 g $L^{-1}$ FW; 8.32±1.55 g $L^{-1}$ DW). The effect of lighting on secondary metabolite contents differed from that on cell growth. For example, dim lamps (at 15 or 30 μmoL $m^{-2}$ $s^{-1}$) enhanced the accumulation of total anthraquinones, phenolics, and flavonoids, while a PPF of 45 μmoL $m^{-2}$ $s^{-1}$ was associated with a reduction of more than two-fold compared with performance at 15 μmoL $m^{-2}$ $s^{-1}$ (Table 4). Although dark-culturing also decreased the contents of secondary metabolites, only slight declines were observed in anthraquinone and phenolics contents (Table 4). In general, dark-culturing is utilized for the production of cell biomass and secondary metabolites (Hahn et al., 2003; Yu et al., 2004) but light also plays an important role in metabolite accumulation. For example, Wu et al. (2007) have reported that the content of caffeic acid derivatives from *Echinacea* adventitious root cultures is enhanced under a 3-h photoperiod at 20 μmoL $m^{-2}$ $s^{-1}$. Paek and Chakrabarty (2003) also have suggested that dim lighting be used for flavonoid production because illumination increases the activities of PAL and Co A-lyase involved in flavonoid biosynthesis. However, Tabata et al. (1974) have demonstrated that light inhibits pigment forma-

**Table 3.** Fresh and dry weights, % dry weight, and growth rate of[y] *Morinda citrifolia* cells as affected by photosynthetic photon flux (PPF) after 3 weeks of bioreactor-culturing.

| PPF (μmoL $m^{-2}$ $s^{-1}$) | Fresh weight ( g $L^{-1}$) | Dry weight ( g $L^{-1}$) | % dry weight | Growth rate (g DW $d^{-1}$) |
|---|---|---|---|---|
| 0 | 160.43±8.82[z] | 8.32±1.55 | 5.18 | 0.4 |
| 15 | 486.24±4.03 | 13.06±0.68 | 2.68 | 1.31 |
| 30 | 465.21±7.02 | 11.79±0.07 | 2.58 | 1.25 |
| 45 | 456.91±4.33 | 8.73±0.23 | 1.88 | 1.23 |

[z]Values are means and standard deviations for 3 replicates.

Table 4. Effect of photosynthetic photon flux (PPF) on secondary metabolite contents in *Morinda citrifolia* cells after 3 weeks of bioreactor-culturing.

| PPF (μmoL $m^{-2}$ $s^{-1}$) | Anthraquinones | | Phenolics | | Flavonoids | |
|---|---|---|---|---|---|---|
| | Total (mg $g^{-1}$ DW) | Yield (mg $L^{-1}$) | Total (mg $g^{-1}$ DW) | Yield (mg $L^{-1}$) | Total (mg $g^{-1}$ DW) | Yield (mg $L^{-1}$) |
| 0 | 16.07±0.18[z] | 133.70 | 14.76±0.74 | 122.80 | 12.69±0.13 | 105.58 |
| 15 | 18.65±0.56 | 243.57 | 21.50±0.79 | 280.79 | 12.51±0.21 | 163.38 |
| 30 | 18.49±0.37 | 217.99 | 19.39±0.46 | 228.61 | 12.35±0.15 | 145.61 |
| 45 | 8.21±0.08 | 71.67 | 8.19±0.31 | 71.50 | 6.92±0.11 | 60.41 |

[z]Values are means and standard deviations for 3 replicates.

tion in cells of *Lithospermum erythrorhizon*.

Previous studies have suggested that lighting conditions can have positive or negative influences on cell growth and secondary metabolite contents. However, the reason for these conflicting effects has not yet been clearly determined. It is assumed that dim lighting creates stress on the cell and acts as an elicitor to stimulate the synthesis of secondary metabolites in *Morinda citrifolia* cell cultures. However, lighting above a certain PPF level disturbs the biosynthetic pathway of secondary metabolites, probably due to excessive oxidative stress (Shohael et al., 2006). Therefore, further studies are required to investigate the relationship between the accumulation of secondary metabolites and the oxidative stress that is induced by illumination.

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