Bacoside biosynthesis during in vitro shoot multiplication in *Bacopa monnieri* (L.) Wettst. grown in Growtek and air lift bioreactor

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Shoot cultures of *Bacopa monnieri* L. (*Brahmi*) were cultivated in shake flask (1 L) and two bioreactor systems: Growtek® (1 L) and modified bench top air lift bioreactor (ALB) (5 L). Continuous immersion and aeration supported excellent growth and enhanced levels of bacoside (A₃+4A₂) production in ALB. Growth index (GI) in terms of dry wt was recorded maximum in the ALB (5.84), followed by Growtek® (4.22) and shake flasks (2.61) after 4 wk of incubation. ALB recorded the highest number of shoots (443.33), as compared to that in Growtek® (42.67) and shake flasks (23.33). Furthermore, bacoside production in shoot cultures of the ALB system was ~ 1.75-fold higher as compared to cultures in shake flask. Nutrient exhaustion in spent medium from the various systems was also measured. Present work reports the feasibility of shoot cultivation in liquid medium to develop a suitable bioreactor strategy.

**Keywords:** Aeration, bacosides, *Bacopa monnieri* (*Brahmi*), continuous immersion, growth index, memory enhancers

**Introduction**

Growing demand for *Bacopa monnieri* (L.) Wettst. (Family: Scrophulariaceae), commonly known as *Brahmi*, and the beneficial effects of its constituents (bacosides) encouraged research into developing efficient methods for controlled cultivation by in vitro approaches. In vitro shoot cultures of *B. monnieri* provide a potential alternative to mass harvesting of plants for the purpose of obtaining bacosides¹. Organ cultures are generally less sensitive to shear stress² and more stable in metabolite yields due to their genetic stability³. Further, the suitability of liquid medium to grow *B. monnieri* has been demonstrated⁴. A recent report describes shoot multiplication of *B. monnieri* using a Growtek® bioreactor⁵. These studies provide a possibility to look for large scale cultivation of shoots using bioreactors.

Bioreactor technology is regarded as a key step for realization of commercial exploitation of plant tissue cultures for propagation and production of phytomedicines. Bioreactor based micropropagation can increase the multiplication rate of cultures and thus reduces the cost, energy and labour requirements in commercial propagation of plants⁶,⁷,⁸. A number of bioreactor configurations have been examined for the growth of shoot culture using liquid medium⁹-¹¹. The earlier attempts⁴,⁵ where feasibility to grow shoots of *B. monnieri* have been demonstrated are silent about bacoside production during cultivation using bioreactors. The present study aims to investigate bacoside biosynthesis during shoot formation in *B. monnieri* grown in bioreactors.

**Materials and Methods**

**Plant Materials and Culture Maintenance**

Shoot cultures of *B. monnieri* were initiated from nodal segments and maintained as described¹. They were grown in 250 mL Erlenmeyer flasks containing Murashige and Skoog (MS)¹² medium supplemented with 3% (w/v) sucrose, 1 mg/L 6-benzylaminopurine (BAP) and solidified with 0.7% (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. The cultures were incubated under controlled growth room conditions of 24±2°C under cool white fluorescent light illumination of 3000 lux at a photoperiod of 16 h. Proliferated shoot cultures were subcultured every 4 wk on liquid medium of the same composition. Multiple shoots cultivated in flasks

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were used as the inoculum for the bioreactor experiments.

Shake Flask Cultivation

A 1 L Erlenmeyer flask (Borosil; OD×ht: 131×220 mm², neck OD 42 mm) was used to grow shoots in liquid medium. The culture flasks were closed tightly with muslin cloth wrapped plugs to guarantee air sterility. The shake flask containing 100 mL liquid MS medium of the above mentioned composition was inoculated with ~ 6.67 excised shoots having ~ 2.54 g fresh wt (FW) and incubated on a rotary shaker (New Brunswick, USA) at 70 rpm in the growth room at a uniform temperature of 24±2°C provided with cool white fluorescent lights (3000 lux) and a 16 h photoperiod (Fig. 1a).

Growtek® (Constant Immersion without Aeration)

A Growtek® (Tarson, India) vessel of 1 L capacity modified into a constant immersion system without aeration by removing the non-absorbing membrane raft from the system was used. It is a low cost autoclavable device (base 100 mm, height 150 mm) made up of polycarbonate with perfect transparency, a side tube (diam 25 mm, length 95 mm) for change of medium and a lid with central and inwardly projected slope for minimizing condensate accumulation inside the vessel. The Growtek vessel containing 100 mL liquid MS medium was inoculated with ~ 9.00 excised shoots having ~ 6.09 g FW and incubated under uniform culture conditions (Fig. 1b).

Air Lift Bioreactor

Bench top air lift modified BioFlo 3000 bioreactor 5 L glass vessel (New Brunswick Scientific, USA) (outer diam 210 mm, inner diam 180 mm, base 180 mm, height 300 mm) supported by a stainless steel stand (diam 280 mm, height 340 mm), air inlet and outlet, sampling ports, accessory connector and a 0.22 µm polytetrafluoroethylene (PTFE) filter (Millipore, MA) connected with silicon tubes of 5 mm diameter was used. Aeration was acquired with sterile air at a rate of 0.5 vvm (air-volume/culture-volume/min) using an air flow control system. Air inlet and exhaust were connected with PALL® disposable filters. Shoot cultures were aerated through a steel sparger (ring shaped, height 260 mm, 1 mm equally spaced diameter openings) at the bottom of the chamber. The inoculation port (outer diam 120 mm, inner diam 90 mm) was covered with a stainless steel lid having a diam of 125 mm. All other ports and accessory connectors were locked for efficient operation. The bioreactor containing 1.5 L of liquid MS medium supplemented with BAP (1 mg/L) was inoculated with ~ 48.33 excised shoots having ~ 24.12 g FW aseptically and maintained under uniform culture conditions (Fig. 1c).
Growth Measurements

Multiple shoots were cultivated for 4 wk in the bioreactor system, harvested and gently blotted dry on filter paper to remove excess water and weighed (FW). Then they were air dried to constant wt and dry weight (DW) was recorded. Growth in terms of growth index (GI=final weight−initial weight/initial weight) on DW basis was calculated for all the treatments. The shoot multiplication rate was calculated as a ratio of number of shoots at the end of the incubation to the initial number of shoots inoculated (Table 1).

Extraction and Analysis of Bacosides

Air dried shoots were powdered and extracted in a soxhlet apparatus with methanol for 5 h at 60°C. The methanolic extract was filtered, air dried and further dissolved in High Performance Liquid Chromatography (HPLC) grade methanol and subjected to HPLC analysis to estimate the total bacosides (A3 & A2). HPLC analysis was performed using an Agilent Technologies Series 1100 (Waldronn, Germany) system equipped with degasser (G 1379A), quaternary pump (G 1311A), auto sampler (G 1313A), column oven (G 1316A), and ultraviolet (UV) detector (G 1314A). HPLC analysis was conducted using acetonitrile and water (48:52, v/v) as the mobile phase with a flow rate of 0.4 mL/min at wavelength of 210 nm on a column RP-18 (E-Merck, 5 µm, 4.0×250 mm). The column temperature was maintained at 30°C for maximum peak efficiency with injection volume of 10 µL and a run time of 30 min for each separation. Data acquisition was performed by Chemstation software A.08.03 (Agilent Technologies, USA). All solvents used for the HPLC analysis were of HPLC grade (acetonitrile, Milli Q distilled water, methanol).

A 1.0 mg/mL stock solution of standard bacoside (mixture of A3 & A2) was prepared in methanol and 1.0 mL of this solution was diluted to 10 mLK with methanol to get a 100 µg/mL working solution. Standard calibration curves were established by plotting the area of peaks against different concentrations of 2, 4, 6, 8 and 10 µL. The linearity curve showed a correlation coefficient of 0.99971 and 0.99980 for bacoside A3 and A2, respectively. Quantification of bacosides in the samples was determined using regression equation of calibration curves. On the basis of the standard curves obtained, the quantity of bacoside A3 and A2 were determined in samples of shoot cultures.

Spent Medium Analysis

Conductivity, pH and nutrient depletion in all the culture systems were measured before commencement of the experiment and at the end of culture cycle of 4 wk. The conductivity (expressed in millie Siemens; mS) was measured using a conductivity meter (Orion 5 star, Thermo Scientific, USA) and the hydrogen ion concentration (pH) was measured using a pH meter (Model Orion 150, Thermo Scientific, USA). Samples from the final medium (the medium after each culture cycle) were taken and analyzed for nutrient exhaustion in the various systems. Nitrogen (N) content in the medium was determined by Kjeldahl method, based on the wet oxidation of organic matter using H2SO4 and a digestion catalyst13. For estimation of total sugar, the medium was taken in a conical flask, in which concentrated HCl and water added in a ratio of 1:3 (v/v). The solution was boiled on a heating plate for 5 min. and then allowed to cool. Few drops of 1% (w/v) phenolphthalein indicator and 40% (w/v) NaOH were added until the solution turned pink. It was acidified with 1 N HCl drop-wise until the pink colour disappeared. The quantity of the sample was measured, taken in a burette and titrated against 10 mL Fehling’s solution to brick red end point using methylene blue as an indicator.

Table 1—Analysis of various bioreactor systems for shoot biomass growth and bacoside production in B. monnieri

<table>
<thead>
<tr>
<th></th>
<th>Shake flask (1 L)</th>
<th>Growtek (1 L)</th>
<th>Air lift bioreactor (5 L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot number</td>
<td>23.33±1.45*</td>
<td>42.67±2.91*</td>
<td>443.33±34.8*</td>
</tr>
<tr>
<td>Shoot length (cm)</td>
<td>2.57±0.09*</td>
<td>4.00±0.36*</td>
<td>8.77±0.43*</td>
</tr>
<tr>
<td>Shoot multiplication rate</td>
<td>3.71±0.77*</td>
<td>4.95±0.96*</td>
<td>9.27±1.1*</td>
</tr>
<tr>
<td>GI (FW basis)</td>
<td>5.56±0.73*</td>
<td>4.90±0.27*</td>
<td>10.65±0.38*</td>
</tr>
<tr>
<td>GI (DW basis)</td>
<td>2.61±0.08*</td>
<td>4.22±0.15*</td>
<td>5.84±1.36*</td>
</tr>
<tr>
<td>Bacoside A3 (mg/g DW)</td>
<td>3.44±0.1*</td>
<td>4.02±0.09*</td>
<td>6.59±0.86*</td>
</tr>
<tr>
<td>Bacoside A2 (mg/g DW)</td>
<td>2.34±0.09*</td>
<td>2.06±0.07*</td>
<td>3.56±0.57*</td>
</tr>
<tr>
<td>Total bacoside (mg/g DW)</td>
<td>5.78±0.17*</td>
<td>6.08±0.16*</td>
<td>10.15±0.28*</td>
</tr>
</tbody>
</table>

Data represents mean±SE of three independent experiments after a culture period of 4 wk
Mean within row followed common letters are not significantly different at P< 0.05, according to Duncan’s Multiple Range Test (DMRT)

*Sum of bacoside A3 and A2
Statistical Analysis

The results are expressed as mean±standard error (SE) of three independent experiments. Data was analyzed by one-way analysis of variance (ANOVA) using statistical software SPSS version 20 (SPSS Inc., Chicago, IL, USA). The significance of differences among means was analyzed using Duncan’s multiple range test (DMRT) at P≤0.05.

Results and Discussion

Growth and Shoot Proliferation

The results obtained for shoot number and biomass production using different systems are presented in Table 1. Maximum GI on dry wt basis was recorded in ALB (5.84), followed by Growtek® (4.22) and shake flask (2.61). An increase in the shoot number from ~48.33 to ~443.33 in ALB system and from ~9.00 to ~42.67 in the Growtek® system was observed. These results can be associated with larger internal space of the ALB with relation to the Growtek®. Growtek culture is practically a closed system, in which gas exchange is slow and, as the culture grows, O_2 content diminishes and CO_2 builds up, which is probably responsible for the lower growth rate. In contrast, ALB system supplied with a constant flow of air, which can keep the dissolved oxygen level high, contributed to a significant increase in the shoot regeneration rate. Higher shoot biomass of *B. monnieri* has been recently reported in Growtek bioreactor when it was supplied with aeration.

Bacoside Production

The total bacoside (sum of A_3 & A_2) content recorded at the end of 4 wk was analyzed. The HPLC profile of shoot biomass produced by ALB is given in Fig. 1f. The maximum content was recorded in shoot biomass cultured in ALB system, which reached 10.15 mg/g DW as compared to 6.08 mg/g DW recorded for Growtek® culture, both of which were higher compared to the shake flask culture (5.78 mg/g DW) (Table 1; Fig. 1d). Bacoside content was ~1.67-fold higher in ALB system to that of shoots grown in Growtek® vessels without aeration. Higher bacoside content indicates that the accumulation of bacosides is associated with aeration provided in the air lift bioreactor system. It is well reported that high aeration improves oxygen transfer, a process that improves growth and secondary metabolite production.

Nutrient parameters

A significant decrease in conductivity of the shake flask culture medium was observed (1.9 mS) as compared to Growtek® (2.54 mS) and bioreactor (3.03 mS) after 4 wks of cultivation. This could be attributed to the utilization of mineral nutrients by shoots for their growth. The pH of the bioreactor medium was adjusted to 5.84, which declined at the end of the culture period (4.18) (Table 2; Fig. 1e). During cultivation, decrease in pH of the medium at the end of the culture period has also been reported in apple rootstock ‘M9 EMLA’. Change in pH can be assigned to nitrogenous absorption by the shoot cultures from the medium. Selective uptake of either nitrate (NO_3^{-}) or ammonium (NH_4^{+}) ions can affect the pH due to differences in proton flow. Higher uptake of NH_4^{+} ions may lead to a decreased pH of the medium due to exudation of H^{+} in the medium. The uptake of both ammonium and nitrate nitrogen started at the initiation of culture and after 4 wk of culture, total nitrogen concentration in bioreactor medium displayed a slight decrease of 0.03% as compared to the shake flask of 0.01%, indicating that the cultures did not consume much nitrogen supplied in the air lift bioreactor system (Table 2). Sugar consumption revealed that, after 4 wk of cultivation in bioreactor, less sugar was consumed from bioreactor medium (1.1%) and Growtek medium (0.3%) in comparison to shake flask cultivation, where most of

<table>
<thead>
<tr>
<th>Nutrient parameters</th>
<th><strong>Control medium</strong></th>
<th>Shake flask (1 L)</th>
<th>Growtek (1 L)</th>
<th>Air lift bioreactor (5 L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.09±0.006</td>
<td>5.37±0.059</td>
<td>4.06±0.03</td>
<td>4.18±0.017</td>
</tr>
<tr>
<td>Conductivity (mS)</td>
<td>5.49±0.023</td>
<td>1.9±0.029</td>
<td>2.54±0.065</td>
<td>3.03±0.036</td>
</tr>
<tr>
<td>Total sugar (%)</td>
<td>2.41±0.018</td>
<td>0.13±0.015</td>
<td>0.30±0.041</td>
<td>1.1±0.015</td>
</tr>
<tr>
<td>Nitrogen (%)</td>
<td>0.05±0.009</td>
<td>0.01±0.0009</td>
<td>0.02±0.0035</td>
<td>0.03±0.0018</td>
</tr>
</tbody>
</table>

Mean within row followed by common letters are not significantly different at P≤0.05, according to Duncan’s Multiple Range Test (DMRT)

**Measurements have been performed on autoclaved medium without culture**
the sugar was consumed by the shoot culture (0.13%). It is possible that the forced aeration of the air lift bioreactor has contributed to the reduction in sugar usage by the explants due to increased photosynthetic capacity.

In conclusion, in vitro cultures of B. monnieri grown in the air lift bioreactor system provide a potential alternative for mass harvesting of shoots for the purpose of bacoside production. These results not only provide a potential alternative strategy for getting quality shoot biomass for controlled production of bacosides, but also serve as an alternative approach for rapid mass cloning of selected genotype of B. monnieri through bioreactor propagation.

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