



Research article

Nexus between light and culture media on morphogenesis in *Bacopa monnieri* and saponin yield thereof

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ABSTRACT

Bacopa monnieri, a well-documented nootropic plant of high commercial global demand had been explored for its effect in alleviating other diseases and symptoms. This is primarily attributed to different phytochemicals present in the plant. One of the major constituents among them are saponins. However, variation in agro-climatic conditions and choice of germplasm often affect the growth rate and yield of phytochemicals that significantly impact the efficacy of the plant and its extract. Tissue culture has been attempted to improve the yield of phytochemicals but is often restricted by higher cost and scalability. Current study explores the role of commercial hydroponic media 'Leafy 200' vis-à-vis Murashige and Skoog (MS) media, under different color and intensity of lights, on plant morphogenesis, biomass and saponin yield. Blue light induced more shoot differentiation than normal white light. Statistical studies performed using fractional factorial design showed no significant variations in the yield of saponins among the extracts. The study suggests that hydroponic culture to be a sustainable solution and possible substitute to tissue culture that may be exploited for scalable cultivation of the plant.

1. Introduction

Bacopa monnieri (Linn.) Wettst., an extensively documented medicinal plant, for its nootropic properties had witnessed a burgeoned interest towards analyses of plant growth, role of culture conditions and elicitation on metabolites yield and their bioactivities (Krishnaraj et al., 2012; Gupta and Sharma, 2019; Komali et al., 2020). The growing disease burden of neurological disorders, especially in developing countries like India (Gourie-Devi, 2014), further augment the need to explore the phyto-pharmacological importance of this plant. Last decade had witnessed extensive studies on the phyto-constituents in the extract of *Bacopa monnieri*, beyond saponin (Muszyńska et al., 2016; Ritter et al., 2020). Such studies are significant, owing to the vast therapeutic application, beyond nootropic ability, exhibited by the phytochemicals of *Bacopa monnieri* (Sukumaran et al., 2019). However, some of the trivial bottlenecks, encountered while cultivation of *Bacopa monnieri* could be the variation in yield of phytochemicals. Such variation often evident in plant growth are primarily due to agro-climatic and genotypic variations along with seasonal variation in the yield of phytochemicals and the bioactivities of extract and herbal formulations (Bhardwaj et al., 2016). Tissue culture techniques have been optimized for the production of

therapeutic phytochemicals rich biomass (Bhardwaj et al., 2017; Bansal et al., 2015) and studies are underway to explore a sustainable scalable solution to mass production of biomass rich in phytochemicals. These concerns obtrude exigencies for exploring alternative robust cultivation strategies. In order to circumvent the impact of cultivation conditions vis-à-vis improving the growth rate and yield of phytochemicals, there is a need to explore some innovative cultivation techniques. Hydroponic culture, also called soil-less culture is obtruding as a well-accepted technique for improving growth and biomass yield in vegetables and herbs (Mattson and Lieth, 2019). Due to the slow, but steady integration and application of hydroponic technique for medicinal plants, scant information highlighting the role of hydroponic cultivation on growth and saponin yield is available. Maneeply et al. (2018) proposed the positive effect of NFT and DFT with higher growth (dry weight) and saponin accumulation than field cultivation, in hydroponic culture of *Bacopa monnieri*, in Hoagland's solution. The need to explore alternative media composition and effect of abiotic factors that may synergistically affecting the growth of *Bacopa monnieri* have not been explored. In current study, the effect of Murashige and Skoog media, 'Leafy 200' hydroponic media and light intensity (and color) were explored, to design a robust bacosides production strategy using hydroponics.

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2. Materials and methods

2.1. Plant material, chemicals, glassware

Bacopa monnieri (Accession No. 14250) (IHBT, Palampur, Himachal Pradesh) was chosen among the available geographical variants, for the study, due to comparatively higher saponin yield, reported previously (Bharadwaj et al., 2017). These plants were maintained in a pot, for a year, within campus premises. The chemicals, growth hormone and basal media were purchased from HiMedia, (India) and methanol (HPLC grade) for phytochemical extraction was purchased from Qualigens Fine Chemicals Pvt. Ltd, (India). The experiments were conducted in phytajars (HiMedia) containing 20 mL of Murashige and Skoog (MS) media. Hydroponic media, used in the study with brand name 'Leafy 200', was purchased from GreenLoop (Pure hydroponic Nutrients). The amount of powdered media used to constitute 1x media solutions, were as per manufacturer's instruction. The saponin standard 'Bacopaside I' used in the study was purchased from Natural Remedies, Bangalore (India). The milliQ water, used in the study, had resistivity of 18.3 Mohms cm^{-1} (conductivity of milliQ water was 0.055 micromhos. cm^{-1}). All chemicals used in the study were of analytical grade, unless specified. All experiments were performed twice and average value with deviations are reported.

2.2. Establishment of inoculum and growth conditions

The excised plant materials, including two nodes with two leaves at each node, were used for propagation of sterile plant material in Murashige and Skoog (MS) medium (basal medium) with 0.8% (w/v) agar. Surface sterilization involved the treatment of explant with 0.1 % (w/v) mercuric chloride (HgCl_2) for 30 s, followed by washing in sterile distilled water. The plant material was further treated with 0.1 % sodium hypochlorite for 40 s and again washed in sterile distilled water. Finally, the explant was washed with 70% (v/v) sterile (filtered) ethanol followed by washing with water. The sterile plants were inoculated on MS Agar media. The media also contained antibiotics viz., ampicillin (100 $\mu\text{g mL}^{-1}$) and amphotericin B (25 $\mu\text{g mL}^{-1}$); and 1mg.L⁻¹ (working concentration) phytohormone BAP (6-Benzylaminopurine), as reported previously (Bharadwaj et al., 2017). The phytajars with sterile plants were propagated in the growth chamber (Vista cell, India) maintained at $25 \pm 2^\circ\text{C}$, illuminated with white light with photoperiod of 12 h.

2.2.1. Effect of light and media composition

Sterile plants, propagated in MS media for 15 days, were used for the study, under different conditions (Table 1). The plantlets containing 2 nodes each were inoculated in different phytajars, with components and light intensity as mentioned in Tables 1 and 2. The *in vitro* propagated sterile plants, in MS agar, were used as inoculant. MS liquid media had same composition as used for sterile plant propagation. The hydroponic

Table 1. Different variables and their amount/intensity, used in fractional factorial design studies for estimating their effect on saponin yield in *in vitro* culture of *Bacopa monnieri*.

Experiment Run No.**	Murashige and Skoog (MS) Media	'Leafy 200' (Hydroponic Media)	Light Intensity
1	1*	-1#	-1
2	-1	1	1
3	1	-1	1
4	-1	1	-1

** The Experimental runs and the matrix were designed using Minitab® 19.

denote lower value of the parameter, used in the experiment (refer Table 2.).

* denote the higher value of the parameter, used in the experiment (refer Table 2.).

Table 2. Different variables and their amount/intensity, used in fractional factorial design studies for estimating their effect on saponin yield in *in vitro* culture of *Bacopa monnieri*.

Variable	Low (-1)	High (1)
Murashige and Skoog	Not added to media	Added to media
Leafy-200 (hydroponic media)	Not added to media	Added to media
Light color and Intensity	Red and Blue (350 lux)	White (2450 lux)

media 'Leafy 200', was prepared as per manufacturer instructions, with electrical conductivity of 152.8 μS . Plants, inoculated in both the media were placed under two different light illumination conditions; white light illumination (2450 lux) and blue/red light illumination (350 lux) for 9 days. All experiments were performed in duplicate and average data was reported.

2.3. Screening of media and light intensity using fractional factorial design

The preliminary study to explore the effect of hydroponic media and light (blue/red and white) on bacosides yield, fractional factorial design was used. The rationale of the study was to explore possible interaction between different components (Table 1) in growth of *Bacopa monnieri* and saponin yield. A 4-run fractional factorial experiment, designed using Minitab® 19, were used to screen three variables at high (+1) and low (-1) levels (Table 2). In the 4-run fractional factorial design (Table 1), each row represents an experiment and each column represents an independent variable.

2.4. Phytochemical extraction

The saponin rich extracts from the plants harvested from different experimental conditions (Table 1.), were prepared using maceration technique, with modifications (Tang et al., 2012). The harvested plants were sun dried and were further subject to the process of maceration in methanol (HPLC grade). The process involved suspension of 2 mg dried *Bacopa monnieri* plant in 1 mL methanol (weight to volume ration of 2:1), followed by shaking at 25 °C for 1 day. The suspensions were centrifuged at 10,000 rpm for 10 min and supernatant was collected and stored at -20 °C for further analysis. The saponin quantification was performed by the method of Hiai et al. (1976), with modifications. The results were documented as yield of saponin estimated as mentioned in Eq. (1).

$$\text{Saponin yield} = \frac{\text{Concentration of saponin in dried plant}}{\text{Amount (weight) of dried plant used for extraction}} \quad (1)$$

2.5. Quantitative estimation of saponins using vanillin sulphuric acid assay

The estimation of saponin is based on structural bioactivity shown by its aglycon (sapogenin) moiety (Hiai et al., 1976). Vanillin reacts with the hydroxyl group (-OH) at C-3 position of Bacopaside I in acidic environment, to form a chromogen.

Quantitative estimation of bacoside was performed using varying concentration (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL⁻¹) of standard compound, Bacopaside I. In the assay, 63 μL of Bacopaside I concentrations were mixed with same volume of 8% Vanillin (w/v in methanol). Further, 625 μL of 72% (v/v) of H_2SO_4 was added and solution was gently mixed. The mixture was incubated in ice for 5 min followed by heating at 60 °C for 10 min. The intensity of the colour was estimated using spectrophotometer (Thermo-scientific Multiscan FC) at 570 nm. The standard plot of Bacopaside I concentration versus absorbance was plotted and used for quantification of saponins in plant extracts. Results were reported as 'mg Bacopaside I equivalent. g dried plant⁻¹'. All the experiments were done in duplicates and average results were reported.

3. Results and discussion

The statistical design of experiment with 4-run experiment was performed and the phytajars with explants were incubated under specified conditions (Figure 1 (A-D)). The harvested plants, after 9-day acclimatization and growth under four different experimental conditions showed significant variations in morphology of the plants (Figure 2). It was observed that propagation of plant in MS liquid media under white and blue/red light showed significant variations in morphology (Figure 2(A) and (C)). Previous studies have reported the photomorphogenesis, due to three main families of photoreceptors, viz. phytochromes, blue-light receptor and less explored UV-B photoreceptors (Jiao et al., 2007). Studies by Najafabadi et al. (2019), highlighted the significance of red light on the growth and hypericins (therapeutically important secondary metabolite) from *in vitro* culture of *Hypericum perforatum*. In another study, Macedo et al. (2011), reported the contrasting effect of red and blue light on the leaf mass density in the tissue culture of *Alternanthera brasiliana*. Studies by Chrysargyris et al. (2017), indicated the effect of nitrogen supplementation during hydroponic culture on plant growth, chlorophyll content and yield of essential oil, highlighting the need of plant specific optimization of the media. Ronga et al. (2018), reported the benefits of digestate (solid and liquid) on dry matter, aromatic compound and sesquiterpenes.

The result in our studies showed more number of leaves and adventitious shoots in hydroponic media illuminated with white light. The leaf area and thickness of the leaves of plant cultured in MS media with white light were more other culture conditions. The explant in MS media under blue/red light showed stunted growth with small leaves (Figure 2(C)). The effect of 'Leafy 200' hydroponic media was also evident in the morphologies of the harvest and showed morphological alternation (Figure 2(B) and (D)). The plant propagated in 'Leafy 200' hydroponic media under white light (Figure 2(B)) showed adventitious rooting from the nodes. Also in comparison to plant propagated under white light in MS media, the number of nodes and leaves in plant in 'Leafy 200' and white light are more. The results suggest the significance of light intensity and media composition on morphogenesis. The results for the first time highlight the effect of light intensity (and color) and commercial hydroponic media on the morphogenesis of *Bacopa monnieri*. Further the effect of culture conditions on total saponin yield as also compared (Table 3).

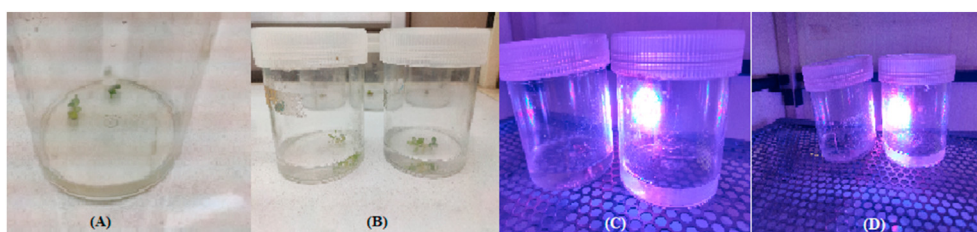


Figure 1. The experimental set-up showing *Bacopa monnieri*, cultured under different experimental conditions; (A) MS + white light, (B) 'Leafy 200' hydroponic media + white light, (C) MS + B/R light, (D) 'Leafy 200' hydroponic media + B/R light.



Figure 2. The morphology of harvested *Bacopa monnieri*, under different experimental conditions; (A) MS + white light, (B) Leafy 200 hydroponic media + white light, (C) MS + B/R light, (D) 'Leafy 200' hydroponic media + B/R light.

Table 3. Results of fractional factorial design experiment (4 run).

Run	Murashige and Skoog Medium [#]	Leafy 200 (hydroponic medium) [#]	Light ^{**}	Saponin yield (Bacopaside-I eq. g dried plant ⁻¹)
1	1	-1	-1	0.00
2	-1	1	1	0.22
3	1	-1	1	0.37
4	-1	1	-1	0.23

[#] For media, -1 is no media and 1 is presence of media.

^{**} Saponin yield (mg Bacopaside I equivalent. g dried plant⁻¹).

3.1. Fractional factorial design for saponin yield

The statistical design of experiment approach using fractional factorial design with 4-runs, to explore the effect of media and light on saponin yield showed insignificant (not detected) yield of saponin in MS media illuminated with white light. The insignificant yield may be due to the low biomass of the harvest. Further analysis of results using Minitab[®] 19. The main effect graph showed insignificant variation in the average saponin yield (Figure 3 (A) & (B)). The main effect of Murashige and Skoog is comparatively more inhibitory than hydroponic media while the effect of light color and intensity is more pronounced. The interactions between the components (Figure 3(B)) was also observed.

The comparison of the yield, analyzed using ANOVA (CI 95%) to test the following two hypotheses;

H₀ (Null): No significant variation in the saponin yield with variations in media and light intensity

H_A (Alternative): Significant variation in the saponin yield with change in media and light intensity

The result of ANOVA (Table 4.) showed insignificant variation in the yield of saponin cultured in both, MS media and hydroponic media ($p > 0.05$). The results suggest that the yield of saponin is not significantly affected on changing the light intensity with either media. The study is important as it suggest hydroponic media 'Leafy 200' to be a cost effect substitute of costly tissue culture technique. Moreover, previous studies have shown that hydroponics or soil-less cultivation have provided a robust solution to the growing concern of variable plant growth or yield

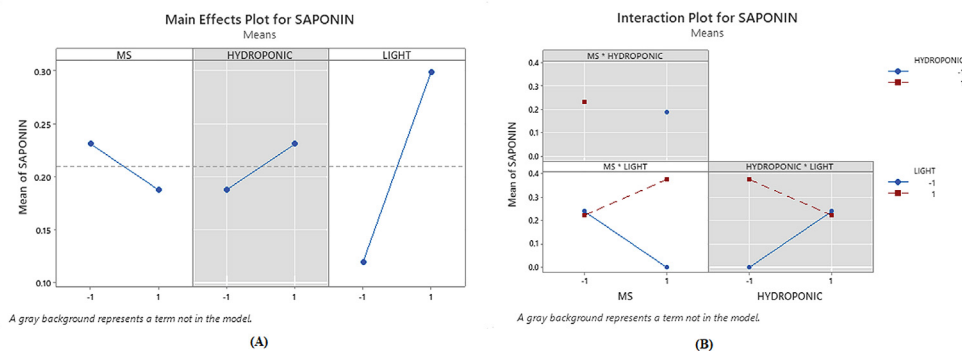


Figure 3. Analysis of the effect of MS media, hydroponic media and light on saponin yield; (A) main effect and (B) interaction plot of MS media, hydroponic media and light intensity on saponin yield. The values -1 and 1 are coded values.

Table 4. ANOVA of MS media and light vis-à-vis Hydroponic media and light.

Source	DF	Adj SS	Adj MS	F-value	p-value*
MS	1	0.001881	0.001881	0.05	0.861
LIGHT	1	0.032175	0.032175	0.84	0.528
Error	1	0.038367	0.038367		
Total	3	0.072424			
HYDROPONIC	1	0.001881	0.001881	0.05	0.861
LIGHT	1	0.032175	0.032175	0.84	0.528
Error	1	0.038367	0.038367		
Total	3	0.072424			

* p-value > 0.05 suggest that Null hypothesis is accepted.

of phytochemicals thereof. Recently, Maneeply et al (2018) reported the high yield of active compounds and higher production of *Bacopa sp.* through hydroponic technique.

The strategy may be extended to further optimize hydroponic cultivation condition of highly demanded nootropic plant *Bacopa monnieri*. Moreover, the study to explore the molecular regulation of media components and light intensity for this high value plant will assist cultivars and farmers to mass cultivate the plant under optimized conditions. The effect of different abiotic components (Table 2) on bacosides yield was observed (Table 3).

4. Conclusion

The analysis of two different media and light intensity and color suggested the possible role of these abiotic factors in morphogenesis and saponin yield. It is expected that other variant of the plant may show deviation from the observation due to insignificant studies on exploring the synergistic effect of hydroponic media and light on the morphogenesis of *Bacopa monnieri*. Moreover, 'Leafy 200', a hydroponic media can be a cost effective substitute for costly tissue culture conditions. The studies underway are focusing towards designing a cost effect hydroponic media for this medicinal plant. The studies may pave the way to develop a sustainable mass cultivation hydroponic system for cultivation of nootropic plant *Bacopa monnieri*.

Declarations

Author contribution statement

Akanksha Aggarwal, Ashwani Mathur: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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