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Original Research Paper

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OPTIMIZATION OF MEDIA AND PH FOR PRODUCTION OF SECONDARY METABOLITES IN *Invitro* CULTURES OF *Bacopa Monnieri* (BRAHMI)

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ABSTRACT Leaf explants of *Bacopa monnieri* were inoculated in MS and B5 medium supplemented with auxin 0.5 mg/l 2, 4 –D. The effect of media adjusted with variable pH (2,4,6,8) was studied on growth and production of secondary metabolites. It was observed that leaf explants cultured in MS and B5 medium supplemented with 0.5 mg/l 2, 4 –D with variable pH significantly affect the callus biomass and secondary metabolites production in *B. monnieri* cultures. MS medium with 2, 4-D (pH-6) and B5 medium with 2, 4-D (pH-4) significantly enhanced the callus biomass production. Whereas for secondary metabolite production, MS medium with 0.5 mg/l 2, 4 –D (pH-8,) was found best for production of alkaloids and pH-4 for phenol production and not significantly affect on flavanoid and saponin. On the other hand, B5 medium with 2, 4-D (pH-6 and 4) was found best for alkaloid and flavanoid whereas pH-8 significantly affect the phenol and saponin production respectively. Both the MS and B5 medium supplemented with 0.5 mg/l 2, 4 –D at pH-2, in which growth was not observed. The study signifies the effect of pH and media composition on the growth and medicinal value of tissue culture of *Bacopa monnieri*.

KEYWORDS : *Bacopa monnieri*, Secondary metabolites, Callus induction,

INTRODUCTION

Bacopa monnieri (L.) Pennell (Scrophulariaceae), commonly known as 'Brahmi' in India, is one of the sources of the medhya rasayan drugs of Ayurveda. *Bacopa monneri* is a small, annual, succulent creeping herb with fleshy leaves. The plant grows in wet, damp and marshy areas. It is distributed in the wet and marshy lands throughout India, Nepal, Sri Lanka, China and Hawaii.The ayurvedic uses of Bacopa on anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome and gastric ulcers[1]. The *in vitro* propagated medicinal plants provide a ready source of biochemical characterization and identification of phyto-constituents[2]. *Bacopa monniera* contained alkaloid brahmine, nicotinine, herpestine, bacosid, triterpenoid saponins, betulinic acid, stigmastanol, -sitosterol, stigmasterol and pseudojujubogenin glycoside[3]. Most secondary metabolites produced by plants exhibit different biological activities, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives[4]. So tissue culture technology has been known as an effective tool to propagate several valuable medicinal plants. Therefore now plant tissue culture has been included as an important tool under biotechnology so that production of metabolites of medicinal value from callus cultures through plant tissue culture paves an efficient way in intervening with the depletion of natural resources thereby, leading to the conservation of endangered plants. Plant cell and tissue culture technique has immense potential to enhance the synthesis and production of secondary metabolites of medicinal importance. Hence this technique was explored in our study to obtain callus biomas and production of secondary metabolites which endows value to the medicinal plants.

MATERIALS AND METHODS

Young and healthy plant of *Bacopa monnieri* were collected from Maharaja Ranjit Singh College of Professional Sciences, Indore (M.P), India. Leaves were used as explants material.

Surface sterilization:

The explants were washed with mild detergent under slow running tap water for 15 min followed by wash in sterile distilled water. The explants were transferred to laminar airflow then surface sterilized with 70% ethanol for 30 second and then the explants were surface sterilized with 0.1% HgCl2 for 2 min and washed thoroughly with sterile distilled water to remove any traces of mercuric chloride. The explants were inoculated on MS medium and B5 medium supplemented with 0.5 mg/l+2, 4-D with varied pH. (2, 4, 6, 8). The cultures were then incubated at 28 ± 2°C under fluorescent tubes in culture room.

Extraction and estimation of secondary metabolites:- Callus biomass was harvested and dried in hot air oven at 50°C then extracted with 70 % alcohol. 10 mg methanolic extract was diluted upto 15 ml distilled water and sample was prepared. Then 1 ml diluted sample was taken into the washed test-tube and 1 ml distilled water was added to make up 2 ml total volume. The test-tubes were kept into oven at 50°C till dried.

Estimation of total alkaloids:- 5 ml phosphate buffer (pH-4.7) was added to dried 1 ml methanolic extract and then 4 ml BCG (Bromocresol green) was added. 4 ml Chloroform was added and mixed well. Now absorbance was taken at 470 nm in spectrophotometer. The quantitative estimation was performed with atropine as standard[5].

Estimation of total flavanoids:- 4 ml distilled water was added to the dried methanolic extract and after 5 minutes 0.3 ml 5% sodium nitrite ($NaNO_3$) was added. Now 0.3 ml 10% Aluminium chloride ($AlCl_3$) was added and after 6 minutes 2 ml 1M NaOH was added following by addition of 2.4 ml distilled water added. Now absorbance was taken at 510 nm in spectrophotometer. The quantitative estimation was performed with Rutin as standard[6].

Estimation of total phenols:- 0.4 ml Folin- Ciocalteu's reagent was added to dried 1 ml methanolic extract and after 5 minutes sodium carbonate solution was added. Then 3.6 ml distilled water was added to it and kept for 90 minutes incubation period. Now absorbance was taken at 750 nm in spectrophotometer. The quantitative estimation was performed with Gallic acid as standard[6].

Estimation of total Saponins:- 1.75 gm (0.7%) vanillin was diluted into 250 ml 65% H_2SO_4 and after cooling 5 ml solution was added to dried 1 ml methanolic extract and cyclomix the sample. Then the tubes were kept in water bath at 65 °C for 1

hour. After 1 hour the reaction was stopped by giving ice (chilled) treatment for 10 min. Now absorbance was taken at 473 nm in spectrophotometer. The quantitative estimation was performed with Bacoside as standard[6].

RESULTS AND DISCUSSION

***In vitro* callus induction:-** The nutrient medium components and the pH of the medium majorly affects the growth and development of plant cells in culture as variation in macro and micro constituents affects the nutritional requirement of the cells and the pH affects the uptake and utilization of the nutrients especially nitrogenous components like NH_4NO_3 etc. Hence the leaf explants were cultured in two majorly used media types that is MS and B5 adjusted with acidic pH (pH 2 and 4), near to neutral pH (pH 6) and basic pH (pH 8) in order to obtain high biomass and high secondary metabolites yield from callus of *Bacopa monnieri*. **(Table 1 and 2)**.

Table 1: Effect of pH on biomass of leaf derived callus of *Bacopa monnieri* cultured in MS medium supplemented with 0.5 mg/l 2, 4 D.

Medium	pH	Fresh weight (gm)	Dry weight (gm)	Moisture Loss (%)
MS + 0.5 mg/l 2,4 D	4	2.98	0.68	338.23
	6	3.13	0.81	286.41
	8	1.80	0.28	542.85

Table 2: Effect of pH on biomasss of leaf derived callus of *Bacopa monnieri* cultured in B5 medium supplemented with 0.5 mg/l 2, 4 D.

Medium	pH	Fresh weight (gm)	Dry weight (gm)	Moisture Loss (%)
B5 + 0.5 mg/l 2,4 D	4	2.41	0.48	402.00
	6	1.99	0.39	410.00
	8	1.21	0.36	236.11

Biomass was yield in MS supplemented with 0.5 mg/l 0.5 2,4-D (pH-6) and B5 supplemented with 0.5 mg/l 0.5 2,4-D (pH-4) compared to all treatment of MS medium and B5 medium with varied pH (2, 4, 6, 8) when the leaf explants inoculated. It was observed that MS medium 0.5 mg/l 0.52,4-D (pH-6) significantly enhanced the maximum callus biomass compared to all treatment of MS and B5 medium supplemented with 0.5 mg/l 0.52,4-D at pH (4) also shows maximum biomass yield compared to all treatment of B5 medium.

Table 5 :- Effect of pH on secondary metabolites production from leaf derived callus of *Bacopa monnieri* cultured in MS medium supplemented with 0.5 mg/l 2 4 D.

Medium	pH	Alkaloid /1gm of crude Extract	% Difference between Natural plant Extract and callus extract	Flavanoid /1gm of crude Extract	% Difference between Natural plant Extract and callus extract	Phenol /1gm of crude Extract	% Difference between Natural plant Extract and callus extract	Saponin /1gm of crude Extract	% Difference between Natural plant Extract and callus extract
MS + 0.5 mg/l 2,4- D	4	0.0168	110.00	0.0003	0.00	0.0268	6.3492	0.0001	0.00
	6	0.0191	138.75	0.0003	0.00	0.0208	0.0000	0.0002	0.00
	8	0.0419	423.75	0.0002	0.00	0.0252	0.0000	0.0002	0.00
Natural Plant Extract	-	0.0080	100%	0.0003	100%	0.0252	100%	0.0002	100%

The leaf explants of *Bacopa monnieri* were inoculated in MS medium with varied pH (2,4,6,8). It was observed that pH-8 was best for alkaloid and pH-4 was phenol production, whereas no significantaly effect on flavanoid and saponin production.

Table 6:- Effect of pH on secondary metabolites production of leaf derived callus of *Bacopa monnieri* cultured in B5 medium supplemented with 0.5 mg/l 2, 4 –D.

Medium	pH	Alkaloid /1gm of crude Extract	% Difference between Natural plant Extract and callus extract	Flavanoid /1gm of crude Extract	% Difference between Natural plant Extract and callus extract	Phenol /1gm of crude Extract	% Difference between Natural plant Extract and callus extract	Saponin /1gm of crude Extract	% Difference between Natural plant Extract and callus extract
B5 + 0.5 mg/l 2,4- D	4	0.0238	197.50	0.0004	33.00	0.0218	0.00	0.0002	0.00
	6	0.0288	260.00	0.0003	0.00	0.028	0.00	0.0002	0.00
	8	0.0055	0.00	0.0003	0.00	0.0293	16.26	0.0003	50.00
Natural Plant Extract	-	0.0080	100%	0.0003	100%	0.0252	100%	0.0002	100%

The leaf explants inoculated in B5 medium supplemented with 2,4-D varied pH (2,4,6,8) it was observed that pH-6 is best for alkaloid, pH-4 is best for flavanoid whereas B5 (pH-8) is best for phenol and saponin production.

Compared to all, it was observed that when leaf explants of *Bacopa monnieri* were inoculated in MS and B5 medium supplemented with 2,4-D varied pH (2,4,6,8). MS medium (pH-8 and pH-4) was best for alkaloid and phenol production, whereas B5 medium (pH-4) was best for flavanoid production and B5 (pH-8) was best for saponin production.

The effect of nutrient medium adjusted with variable pH (2,4,6,8) was studied on production of secondary metabolites and it was observed that MS medium + 0.5 mg/l 2,4-D (pH-8 and pH-4) was significantly affect on alkaloid and phenol production respectively, whereas B5 medium + 0.5 mg/l 2,4-D (pH-4 & pH-8) was also significantly affect for flavanoid and saponin production.

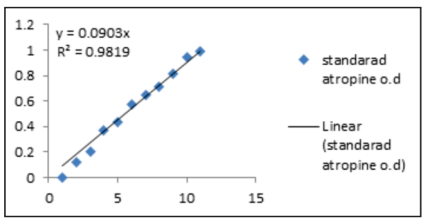

Fig 4:- Variation of absorbance with atropine concentration at 470 nm

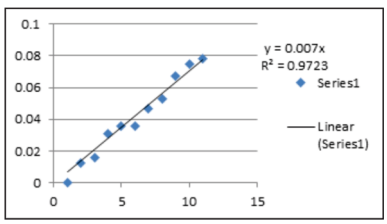

Fig 5:- Variation of absorbance with rutin concentration at 510 nm

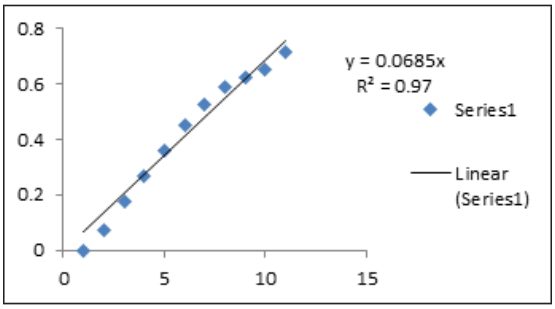

Fig 6:- Variation of absorbance with gallic acid concentration at 750 nm

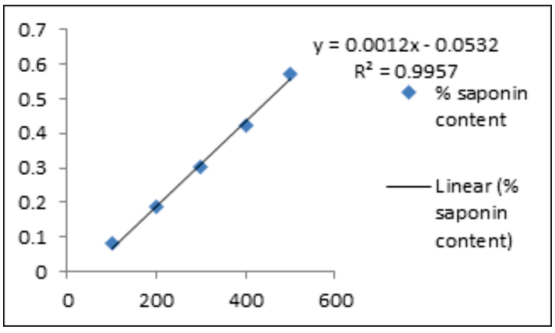

Fig 7:- Variation of absorbance with Bacosid concentration at 473 nm

CONCLUSION

Leaf derived callus from *Bacopa monnieri* were grown in both MS and B5 medium with varied pH (2,4,6,8). It was found that *Bacopa monnieri* cultures grew better on both MS and B5 medium supplemented with 2,4-D. In *in-vitro* cultures of Bacopa on MS medium supplemented with 2,4-D (pH-6) was best in callus biomass production compare to all treatment of MS whereas B5 medium (pH-4) supplemented with 2,4-D was also found best for callus production compare to all treatment of B5. On other hand the production of secondary metabolites on different medium at varied pH was also observed. MS medium supplemented with 2,4-D (pH-8 and 4) significantly enhanced the alkaloid and phenol production compared to all treatment of MS whereas B5 medium (pH-6) is better for alkaloid production and pH-4 was best for flavanoid as well as B5 medium with pH-8 was also found best for phenol and seponin production.

Compare to all the treatment of MS and B5 supplemented with 2,4-D at varied pH, it was observed that MS 2,-D (pH-8 and pH-4) was best for maximum alkaloid production, whereas B5 medium with pH-4 best for flavanoid and B5 medium with pH-8 and pH-4 was best for maximum phenol and saponin production and medicinal value of tissue culture of *Bacopa monnieri*.. But both the MS and B5 medium supplemented with 0.5 2,4-D at pH-2, growth was not observed. Future studies can be targeted on standardization of large scale production of secondary metabolites to increased demand in the area of neuropharmacology.

Medium	pH	Alkaloid (mg/gm)	Flavaloid (mg/gm)	Phenol (mg/gm)	Saponin (mg/gm)
B5+0.5 2,4,-D	4		0.0004		
	8			0.0293	0.0003
MS+0.5 2,4,-D	8	0.0419			
Natural Plant Extract		0.0080	0.0003	0.0252	0.0002

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