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Effects of Adenine Sulphate and Casein Hydrolysate on Production of Callus Biomass and Accumulation of Bacoside in *Bacopa monnieri*

AVNI DAHIYA[1], ADHISHA DAHIYA, SUBHASH KAJLA[2] AND NAMITA SINGH*

Department of Biotechnology, Guru Jambeshwar University of Science & Technology, Hisar-125 001 (Haryana), India

**(e-mail: namitasingh71@gmail.com; Mobile: 94169 28883)*

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ABSTRACT

Bacopa monnieri (L.) Wettst., a medicinally important and endangered herb rich in the nootropic compound bacoside-A, faces severe constraints in conventional propagation due to poor seed viability and overharvesting of wild populations. This study developed an optimized *in vitro* protocol to enhance callus induction, biomass production and bacoside-A accumulation. Leaf explants from *in vitro* grown plants were cultured on Murashige and Skoog (MS) medium with varying plant growth regulators (PGRs), where a combination of 1.5 mg/l BAP and 2.5 mg/l NAA produced the highest callus induction (90%) within 12 days. Callus proliferation and secondary metabolite synthesis were further improved by supplementing the medium with adenine sulphate (ADS) and casein hydrolysate (CH), individually and in combination. The synergistic treatment of 50 mg/l ADS and 100 mg/l CH yielded the maximum biomass (0.294 g dry weight) and bacoside-A content (15.75 mg/g DW in ethanol extract), significantly surpassing control and single-additive treatments. Ethanol consistently proved superior to aqueous extraction for bacoside-A recovery. These findings demonstrated that the strategic use of optimized PGRs with ADS and CH can greatly enhance *in vitro* propagation and metabolite production in *B. monnieri,* offering a scalable approach for conservation and commercial exploitation of this high-value medicinal plant.

Key words: *Bacopa monnieri*, callus induction, bacoside-A production, adenine sulphate (ADS), casein hydrolysate (CH)

INTRODUCTION

Bacopa monnieri has been extensively used in the traditional Indian medicine systems for its cognition improving abilities owing to triterpenoid saponin bacosides (Kharde *et al*. 2018). It has a long history of use in the treatment of a variety of ailments, such as epilepsy, anxiety, memory enhancement, lack of concentration, stroke, sedative, mental diseases, etc. (Srivastava *et al.,* 2019). In Ayurveda, it has been used as a brain tonic to improve memory, concentration and learning capacity and also to cure mental illness (Brimson *et al*. 2021; Lopresti *et al*. 2021). Several ancient Ayurvedic scriptures like *Charaka Samhita*, *Sushrutha Samhita* and *Astanga Hrdaya* have documented numerous medicinal benefits of Bacopa like a memory and intelligence enhancer, antidepressant agent, analgesic, cardio-tonic and many more. A range of biochemical compounds has been identified from the alcoholic extracts of *B. monnieri* including dammarane-type triterpenoid saponins known as bacosides, alkaloids (nicotine, brahmine), cucurbitacins, sterols (stigma-sterol, b-sitosterol), D-mannitol and betulinic acid. Out of these, the best studied is a saponin called Bacoside-A, which comprises Bacoside-A3, bacopaside II, bacopasaponin C together with a jujubogenin isomer of bacosaponin C and is attributed with the nootropic/memory related function (Kharde *et al*., 2018; Dey *et al*., 2019; Banerjee *et al*., 2021; Nandy *et al*., 2022).

The vegetative propagation in Brahmi is very slow and seed propagation is also very difficult due to poor propagule viability and frequent seedling mortality. Thus, traditional propagation has numerous downsides, such as being laborious, seasonal in terms of flowering and fruiting and time-demanding. To meet the

[1]Presently at: Crop Improvement Division, ICAR-Central Soil Salinity Research Institute, Karnal-132 001 (Haryana), India.

[2]Department of Botany and Plant Physiology, CCS Haryana Agricultural University, Hisar-125 001 (Haryana), India.

raw material requirements as well as to preserve this medicinally essential plant, development and standardization a specialized propagation protocol for production of large quantity of disease-free plants was necessitated. Here, biotechnological interventions, specifically micropropagation techniques, have proved to be an effective alternative ensuring conservation of natural population along with enhanced biomass and secondary metabolites production for commercial use (Kharde *et al*., 2018; Sharma *et al*., 2019; Koul and Mallubhotla, 2020).

B. monnieri is the main natural source of bacosides, but heavy collection by pharmaceutical industries has caused a sharp decline in its wild populations. This overharvesting has placed the plant under the category of rare and endangered species, raising concerns about loss of genetic resources. Several studies have focused on improved production of secondary metabolites, both in whole plants as well as *in vitro* cultured tissues which offer the foundation for the commercial use of *in vitro* culture techniques. The production of secondary metabolites can be significantly increased through approaches such as elicitation (Koul and Mallubhotla, 2020) modification of plant growth regulators (Sanputawong *et al*., 2021), optimization of culture conditions (Dey *et al*., 2019), precursor feeding (Muszyñska *et al*., 2016), micropropagation, use of bioreactors (Sharma *et al*., 2019) and supply of carbon (Fink *et al*., 2018), and nitrogen source (Muszyñska *et al*., 2016; Dey *et al*., 2020).

Incorporation of adenine sulphate and casein hydrolysate in the nutrient medium can be one of the strategies for optimal biomass production as well as accumulation of bacoside-A in *B. monnieri*. Adenine sulphate, an adenine derivative, is known to support plant tissue growth and promoting axillary shoot proliferation in shoot cultures, and inducing adventitious shoot formation either directly from explants or indirectly through callus (Shukla *et al*., 2023). Research also indicates that organic nitrogen sources, such as amino acids, are often more effective than inorganic sources like nitrates or ammonium salts in enhancing cell proliferation and regeneration in certain genotypes. Furthermore, mixtures of amino acids, such as casein hydrolysate, have been found to be especially beneficial for shoot multiplication (Kharde *et al*., 2018). Therefore, in the present study, the effect of supplementation of ADS and CH through nutrient medium on the production of callus biomass and accumulation of bacoside-A in *B. monnieri* was studied.

MATERIALS AND METHODS

Healthy leaf explants were used for friable callus induction on MS media**.** To minimize seasonal variations and to prevent contamination in callus cultures, leaf explants measuring 0.5-0.7 cm from the *in vitro* propagated plants maintained in a controlled culture environment were used for callus induction and culture optimization in MS medium solidified with 0.8% agar, supplemented with various growth hormones. The cytokinins used were BAP and KIN, whereas the auxins used were IAA and NAA. These growth hormones of plants were used either alone or in different combinations with each other. The MS basal medium without having any growth hormone served as a control. The medium's pH was adjusted to 5.8 using either 1 N sodium hydroxide (NaOH) or 1 N hydrochloric acid (HCl) before being sterilized through autoclaving at 121°C and 15 lb for 20 min. All the cultures were incubated at 25±2°C under an 8-h photoperiod provided by cool white fluorescent tubes. Sub culturing of the callus was performed every 28 days. The MS basal medium without any growth hormones did not support callus formation. The per cent callus induction, number of days required for callus induction and visual index to observe colour, shape and texture of the callus formed, mean number of shoots per explant, average length of shoot and mean number of nodes per shoot was observed after six weeks of culture, to select the most suitable PGR regime.

Callus growth was further enhanced by incorporating two additive, adenine sulphate and casein hydrolysate, either individually or in various combinations, into MS media supplemented with the most effective growth regulators from the previous callus induction experiment (1.5 mg/l BAP and 2.5 mg/l NAA). Adenine sulphate was added to the medium at concentrations of 25, 50, 75 and 100 mg/l, while casein hydrolysate was introduced at concentrations of 50, 100, 150 and 200 mg/l

in separate set of cultures. Additionally, four different combinations of these additives were tested to assess their synergistic effect (Table 2.). Cultures without additive treatment served as controls. After six weeks of initiation of culture, the callus was harvested, and growth was measured in terms of dry weight by oven-drying the biomass at 60°C until a constant weight was reached. The quantification of secondary metabolite (bacoside-A) was done by comparing HPLC retention times with those of standard reference compounds.

The dried samples were crushed and ground to a powdered form with the help of sterilized pestle mortar. The crushed samples (100 mg) were subjected to ethanol and water extraction using a Soxhlet apparatus. The extract was filtered and dried. Later the dried extract powder was re-dissolved in HPLC grade methanol and filtered through a 0.22 pm millipore membrane before subjecting to quantitative analysis using HPLC. The quantification of bacoside-A was done by comparing the retention time and spectral data with those of standard compound. Calibration curve with the standard was developed with diluted samples of standard compound. The bacoside-A was analyzed using HPLC system equipped with RID detector (RID-20A, Shimadzu, Japan), an auto-sampler, Phenomenex, C_{18} (4.6 × 250 mmi.d., 5 ìm particle size) and a Lab-solution software. The mobile phase solvent A was sodium acetate buffer and solvent B was acetonitrile (65:35) at flow rate of 1 ml/min having column temperature at 25°C. The detection wavelength was set at 345 nm. The injection volume was 10 µl.

The experiments on biomass production and accumulation of bacoside-A were set up in completely randomized design (CRD) in triplicate. The data were analyzed by analysis of variance (ANOVA) followed by Duncan multiple range test. Data analysis was carried out by using R software.

RESULTS AND DISCUSSION

The results pertaining to callus induction are presented in Table 1. Leaf explants from *in vitro* grown plants were used for callus induction to reduce seasonal variability and maintain aseptic conditions. Explants cultured on basal MS medium without plant growth regulators (PGRs) did not show any response, confirming that endogenous hormones alone could not support dedifferentiation and proliferation, thus highlighting the need for exogenous PGRs in callus induction. Similarly, when auxins (IAA, NAA) or cytokinins (BAP, KN) were used individually at concentrations between 0.5 and 2.5 mg/l, no callus formation occurred. This clearly indicated that a balanced supply of both auxin and cytokinin was essential to disturb auxin polarity and reprogram cells into a dividing state (Fehér, 2023).

The combination of BAP and NAA proved to be the most effective among all treatments. A medium containing BAP (1.5 mg/l) with NAA (2.5 mg/l) showed the highest callus induction (90%) within 12 days (treatment C_{18}), while BAP (1.5 mg/l) with NAA (1.5 mg/l) gave 80% induction in 14 days (treatment C_{17}). The calli obtained were light green and firm. In contrast, media containing KN with IAA produced poor results, with only 0-20% callus induction after 22 days, and the calli were mostly brown and hard. These findings suggest that not all auxin–cytokinin combinations are equally effective and that BAP plays a more important role than KN in inducing callus in *B. monnieri*. Earlier studies have also shown that BAP is superior to KN for callus induction in Bacopa (Ranjan *et al*., 2018) and other plant species including *Orthosiphon stamineus* (Yoong *et al*., 2019), *Atropa acuminata* (Dar *et al*., 2021) and Aglaonema ‘Siam Aurora’ (Anjani and Ratnawati, 2023).

The role of auxins in callus induction is particularly critical, as they are known to reprogram differentiated cells into a dedifferentiated, dividing state (Fehér, 2023). Cytokinins, when combined with auxins, act additively by enhancing tissue proliferation and stability. However, any variation in concentration reduced callus growth, suggesting that explants require an optimum hormonal balance for successful induction. This optimum may further depend on factors such as genotype, explants source and physiological condition of the donor plant.

Overall, the results clearly established that callus induction in *B. monnieri* required an exogenous supply of both auxins and cytokinins, with BAP (1.5 mg/l) and NAA (2.5 mg/l) being the most effective combination for rapid and high-frequency callus induction.

To enhance callus proliferation, adenine sulphate (ADS) and casein hydrolysate (CH)

Table 1. Effect of various plant hormones on callus establishment

Media code	Callus initiation (days)	Callus response (%)	Visible characteristics of callus		
			Growth	Colour	Texture
C_0 (Control)	-	0	-	-	-
C_1 (BAP 0.5)	-	0	-	-	-
C_2 (BAP 1.5)	-	0	-	-	-
C_3 (BAP 2.5)	-	0	-	-	-
C_4 (KN 0.5)	-	0	-	-	-
C_5 (KN 1.5)	-	0	-	-	-
C_6 (KN 2.5)	-	0	-	-	-
C_7 (IAA 0.5)	-	0	-	-	-
C_8 (IAA 1.5)	-	0	-	-	-
C_9 (IAA 2.5)	-	0	-	-	-
C_{10} (NAA 0.5)	-	0	-	-	-
C_{11} (NAA 1.5)	-	0	-	-	-
C_{12} (NAA 2.5)	-	0	-	-	-
C_{13} (BAP 0.5 + NAA 0.5)	17	70	++	Light green	Hard
C_{14} (BAP 0.5 + NAA 1.5)	14	80	+++	Light green	Hard
C_{15} (BAP 0.5 + NAA 2.5)	16	70	++	Light green	Hard
C_{16} (BAP 1.5 + NAA 0.5)	15	70	++	Light green	Hard
C_{17} (BAP 1.5 + NAA 1.5)	14	80	+++	Light green	Hard
C_{18} (BAP 1.5 + NAA 2.5)	12	90	++++	Light green	Hard
C_{19} (BAP 2.5 + NAA 0.5)	0	0	-	-	-
C_{20} (BAP 2.5 + NAA 1.5)	27	70	++	Light green	Hard
C_{21} (BAP 2.5 + NAA 2.5)	0	0	-	-	-
C_{22} (KN 0.5 + NAA 0.5)	22	50	+	Yellowish	Hard
C_{23} (KN 0.5 + NAA 1.5)	14	70	++	Yellowish	Hard
C_{24} (KN 0.5 + NAA 2.5)	26	40	+	Pale yellow	Hard
C_{25} (KN 1.5 + NAA 0.5)	16	70	++	Yellowish	Hard
C_{26} (KN 1.5 + NAA 1.5)	18	70	++	Yellowish	Hard
C_{27} (KN 1.5 + NAA 2.5)	22	70	++	Yellowish	Hard
C_{28} (KN 2.5 + NAA 0.5)	28	60	+	Light green	Hard
C_{29} (KN 2.5 + NAA 1.5)	-	0	-	-	-
C_{30} (KN 2.5 + NAA 2.5)	-	0	-	-	-
C_{31} (BAP 1.5 + IAA 0.5)	26	60	+	Brownish	Hard
C_{32} (BAP 1.5 + IAA 1.5)	-	0	-	-	-
C_{33} (BAP 1.5 + IAA 2.5)	-	0	-	-	-
C_{34} (KN 2.5 + IAA 0.5)	29	10	+	Brownish	Hard
C_{35} (KN 2.5 + IAA 1.5)	22	20	+	Brownish	Hard
C_{36} (KN 2.5 + IAA 2.5)	-	0	-	-	-

- No response.

were incorporated into Murashige and Skoog (MS) medium already optimized with 1.5 mg/l BAP and 2.5 mg/l NAA. Treatments with these additives were compared against a control lacking additives. After six weeks of culture, calli were harvested and oven-dried at 60°C to constant weight for biomass quantification.

The addition of ADS and CH, individually or in combination, significantly influenced callus growth (Table 2, Fig 1). Statistical analysis (one-way ANOVA followed by Duncan's multiple range test at P d" 0.05) confirmed significant differences among treatments. Among the ADS treatments, 50 mg/l (EC_2) gave the highest biomass (0.243±0.013 g), a 51.85% increase over the control (0.160±0.020 g). Higher ADS concentrations (75 and 100 mg/l; EC_3 and EC_4) caused a moderate decline relative to 50 mg/l but still exceeded the control.

CH supplementation also showed a dose-dependent effect. The 150 mg/l treatment (EC_7) produced the maximum biomass among CH-only treatments (0.281±0.017 g; 75% over control), whereas 200 mg/l (EC_8) reduced biomass to 0.183±0.029 g, indicating a threshold beyond which CH became inhibitory. The strongest effect was achieved with combined addition: 50 mg/l ADS+100 mg/l CH

Table 2. Effect of additives on callus biomass of *B. monnieri* harvested after six weeks

Media code	Growth regulators (mg/l)		Additives (mg/l)		Dry wt. of callus (g)	Increase in dry weight of callus over control (%)
	BAP	NAA	Adenine sulphate	Casein hydrolysate		
EC_0 (Control)	1.5	2.5	-	-	0.160±0.020^{f}	-
EC_1	1.5	2.5	25	-	0.172±0.021^{ef}	7.50
EC_2	1.5	2.5	50	-	0.243±0.013^{abcd}	51.85
EC_3	1.5	2.5	75	-	0.203±0.019^{cdef}	26.87
EC_4	1.5	2.5	100	-	0.183±0.013^{def}	14.37
EC_5	1.5	2.5	-	50	0.252±0.020^{abc}	57.50
EC_6	1.5	2.5	-	100	0.224±0.029^{bcdef}	40.00
EC_7	1.5	2.5	-	150	0.281±0.017^{ab}	75.00
EC_8	1.5	2.5	-	200	0.183±0.029^{def}	12.50
EC_9	1.5	2.5	50	100	0.294±0.042^{a}	83.75
EC_{10}	1.5	2.5	50	150	0.240±0.005^{abcd}	50.00
EC_{11}	1.5	2.5	75	100	0.214±0.019^{cdef}	33.75
EC_{12}	1.5	2.5	75	150	0.231±0.012^{abcde}	44.37

Values represent the mean of three replicates±SE. The same letters within columns are not significantly different, according to Duncan's multiple range ($P \leq 0.05$) test.

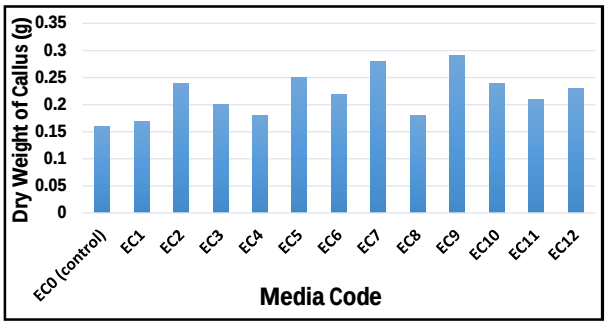

Fig. 1. Effect of adenosine sulphate and casein hydrolysate on callus biomass production.

(EC_9) produced the highest dry weight (0.294±0.042 g), an 83.75% increase over control, surpassing all other treatments and suggesting a synergistic interaction. Other combinations such as 50 mg/l ADS+150 mg/l CH (EC_{10}) and 75 mg/l ADS with 100 or 150 mg/l CH (EC_{11}, EC_{12}) also significantly enhanced biomass but to a lesser extent than EC_9. Based on these findings, the combination used in EC_9, along with optimal BAP and NAA, was recommended for promoting callus proliferation in *B. monnieri* under the conditions tested.

These observations align with previous reports on the benefits of organic additives and purine derivatives in plant tissue culture. ADS, a purine derivative and cytokinin precursor, enhances cell division and shoot proliferation (Shukla *et al*., 2023). In papaya callus cultures, 50 mg/l ADS significantly increased shoot numbers per explant (Intanu *et al*., 2023), while in *Simmondsiav chinensis*, ADS improved shoot multiplication (Raman Bala *et al*., 2020). CH provides amino acids, peptides, vitamins, and minerals that improve nutrient availability and metabolic activity in plant tissues. In many studies, casein hydrolysate was used to supplement the liquid MS medium for suspension culture to produce maximum bacoside (Kharde *et al*., 2018). It enhanced callus growth in hazel (*Corylus avellana* L.) at 1000 mg/l (Salehi *et al*., 2017) and improved nitrogen uptake and metabolism in *Nicotiana tabacum* (Belonozníkova *et al*., 2023).

The synergistic effect of ADS and CH in the present study can be attributed to their complementary roles: ADS may increase endogenous cytokinin levels, thereby stimulating cell division, while CH supplies essential amino acids and peptides to support protein synthesis and metabolic activity. This dual action created a favourable environment for enhanced callus proliferation and biomass accumulation.

In summary, incorporating 50 mg/l ADS and 100 mg/l CH into MS medium supplemented with 1.5 mg/l BAP and 2.5 mg/l NAA markedly improved callus biomass in *B. monnieri*. This optimized medium formulation offered a promising approach for large-scale in vitro

Table 3. Effect of additives on bacoside A content of *B. monnieri* harvested after six weeks

Media code	Elicitors (mg/l)		Bacoside A content (mg/g DW)			
	Adenine sulphate	Casein hydrolysate	Ethanol extract		Aqueous extract	
			Content	Increase as compared to control (fold)	Content	Increase as compared to control (fold)
EC_0	-	-	5.14±0.151^{g}	-	4.35±0.104 ^{g}	-
EC_1	25	-	8.38±0.412^{f}	1.63	5.48±0.050 ^{f}	1.26
EC_2	50	-	12.50±0.333^{b}	2.43	7.27±0.055 ^{d}	1.67
EC_3	75	-	11.23±0.242^{cd}	2.18	8.74±0.142 ^{c}	2.01
EC_4	100	-	10.20±0.078^{de}	1.98	7.64±0.119 ^{d}	1.75
EC_5	-	50	7.41±0.137^{f}	1.44	5.23±0.045 ^{f}	1.20
EC_6	-	100	9.59±0.046^{e}	1.86	7.61±0.253 ^{d}	1.75
EC_7	-	150	11.78±0.125^{bc}	2.29	7.81±0.046 ^{d}	1.80
EC_8	-	200	10.55±0.230^{de}	2.05	6.50±0.094 ^{e}	1.49
EC_9	50	100	15.75±0.142^{a}	3.06	10.63±0.256 ^{b}	2.44
EC_{10}	50	150	14.86±0.154^{a}	2.89	13.27±0.098 ^{a}	3.05
EC_{11}	100	100	12.81±0.195^{b}	2.49	11.01±0.179 ^{b}	2.53
EC_{12}	100	150	12.20±0.130^{bc}	2.37	12.85±0.115 ^{a}	2.95

Values represent the mean of three replicates±SE. The same letters within columns are not significantly different, according to Duncan's multiple range ($P \leq 0.05$) test.

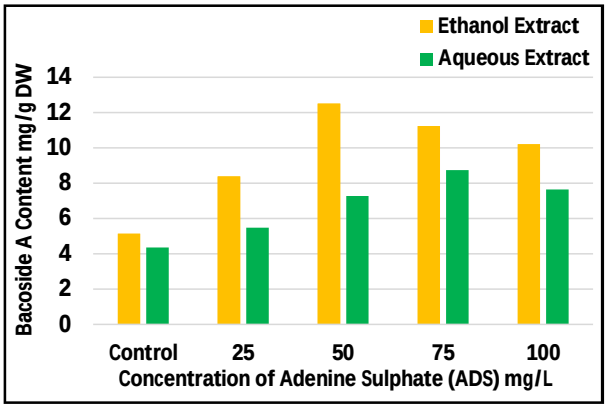

Fig. 2A. Effect of adenine sulphate (ADS) on bacoside A content in callus cultures of *B. monnieri.*

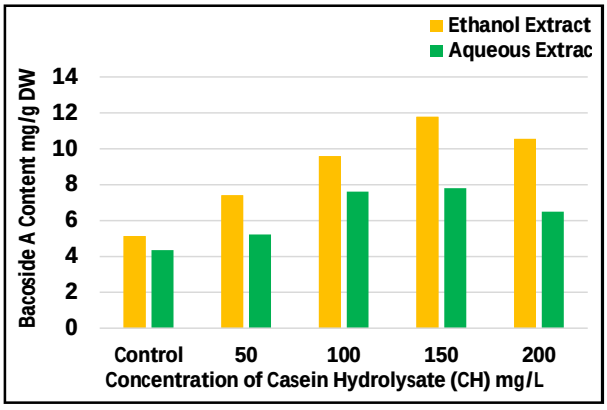

Fig. 2B. Effect of casein hydrolysate (CH) on bacoside A content in callus cultures of *B. monnieri.*

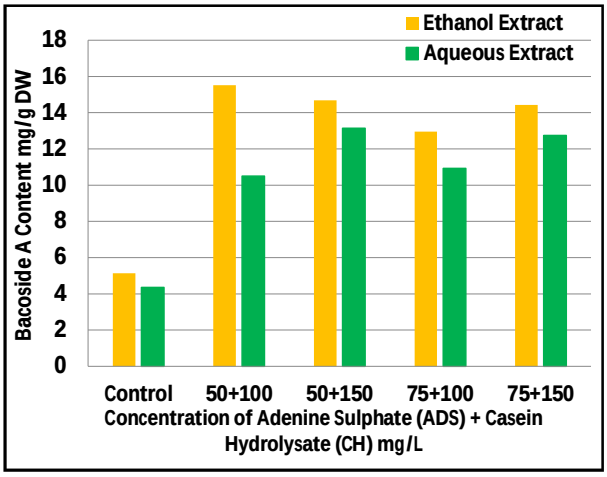

Fig. 2C. Effect of adenine sulphate (ADS)+casein hydrolysate (CH) on bacoside A content in callus cultures of *B. monnieri.*

propagation and biomass production in this medicinal plant.

The adenine sulphate (ADS) and casein hydrolysate (CH), individually or together, influenced bacoside A production in *B. monnieri* callus cultures (Table 3 and Fig. 2A, 2B, and 2C). Ethanol consistently extracted more bacoside A than aqueous methods across all treatments confirming its superior extraction efficiency.

In untreated callus (EC_0), bacoside A content was low, providing a baseline for evaluating elicited cultures. Addition of ADS alone (EC_1-

EC_4) produced a concentration dependent increase in bacoside A, with a maximum at mid level concentrations (50-75 mg/l), followed by a decline at 100 mg/l. This pattern suggested that moderate ADS levels stimulated secondary metabolism, whereas higher concentrations may induce feedback inhibition. Similarly, CH supplementation (EC_5 - EC_8) enhanced bacoside-A accumulation up to 150 mg/l but showed a slight decline at 200 mg/l, indicating an optimal range for stimulation.

The most striking effect was observed when ADS and CH were combined (EC_9-EC_{12}). The combination of 50 mg/l ADS and 100 mg/l CH (EC_9) produced the highest bacoside A levels in ethanol (15.75±0.142 mg/g DW) and aqueous extracts (10.63±0.256 mg/g DW). EC_{10} (50 mg/l ADS+150 mg/l CH) yielded the highest aqueous extract values but slightly lower ethanol yields compared to EC_9. Higher ADS+CH combinations (EC_{11} and EC_{12}) maintained elevated bacoside A but at lower levels than EC_9 and EC_{10}. These results confirmed a synergistic interaction between ADS and CH at optimal concentrations, significantly surpassing the effects of either additive alone.

ADS is a purine derivative and cytokinin precursor known to promote cell division and enhance shoot or metabolite production in various plant systems (Shukla *et al*., 2023). In the present study, ADS alone increased callus proliferation and bacoside A up to 50 mg/l but declined thereafter, a typical dose response pattern reported in plant cell cultures. CH, rich in amino acids and peptides, is an organic nitrogen source that accelerates cell growth and secondary metabolite synthesis (Kharde *et al*., 2018). Similar positive effects of CH on callus growth and metabolite production have been reported in *B. monnieri* and other species including Abelmoschus (Melvin *et al*., 2018). At levels above 150 mg/l, CH also showed reduced stimulatory effects, likely due to saturation or feedback mechanisms (Ramirez-Estrada *et al*., 2016).

The synergistic action of ADS and CH resulted from their complementary roles. ADS enhanced cytokinin like signalling and cell division, while CH supplied readily available nitrogen and amino acids, stimulating primary metabolism and increasing the flux into the bacoside biosynthetic pathway. This dual mechanism explains the significant increase in bacoside A observed with combined treatments. Previous studies have reported similar synergistic effects of multiple additives on secondary metabolite accumulation in *B. monnieri* and other plant species (Seth *et al*., 2020).

Overall, the findings demonstrated that targeted elicitation using ADS and CH, particularly at mid-level concentrations, significantly boosted bacoside A production in *B. monnieri* callus cultures. These results support the potential application of optimized additive combinations and extraction methods for large scale production of bacoside A and related phytopharmaceuticals.

CONCLUSION

The present study established an efficient and reproducible system for callus induction, proliferation and bacoside A enhancement in *Bacopa monnieri* using leaf explants. MS medium supplemented with 1.5 mg/l BAP and 2.5 mg/l NAA produced the highest and fastest callus induction, while the addition of 50 mg/L adenine sulphate and 100 mg/l casein hydrolysate markedly increased both biomass and bacoside A content, demonstrating a strong synergistic effect. This optimized protocol provides a reliable platform for large scale *in vitro* propagation and metabolite production. Future work can build on these findings by regenerating complete plantlets from the optimized callus, scaling up cultures in bioreactors, testing other additives, and integrating molecular or metabolic engineering approaches to further enhance bacoside biosynthesis and ensure genetic stability for phytopharmaceutical applications.

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