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Research Article

Optimization of cytokinin-mediated *in vitro* shoot induction and multiplication in *Bacopa monnieri* (L.) Wettst. using nodal explants

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Abstract
Bacopa monnieri (L.) Wettst., commonly known as Brahmi, is a valued medicinal plant in Ayurveda, recognized for its memory-enhancing and stress-relieving properties, largely due to its bioactive compounds, including bacosides, alkaloids, flavonoids, and phenolics. The present study aimed to optimize *in vitro* shoot induction and multiplication from nodal explants of the species *B. monnieri*.Explants were cultured on Murashige and Skoog (MS) medium supplemented with varying concentrations of cytokinins, 6-benzylaminopurine (BAP) and kinetin, applied individually or in combination.BAP at 2.5 mg/L induced 58.3% regeneration with 7.0 ± 0.3 shoots per explant and 2.2 ± 0.9 cm shoot length, whereas kinetin at 2.0 mg/L yielded 50.0% regeneration with 6.0 ± 1.8 shoots and 2.1 ± 1.4 cm shoot length. The combined application of 1.0 mg/L BAP + 1.5 mg/L kinetin produced the highest shoot induction (66.6%), generating 8.0 ± 0.9 shoots per explant with 2.6 ± 1.9 cm shoot length, but post-hoc comparison revealed that shoot number did not differ significantly from the best individual BAP treatment ($p > 0.05$). Thus, the combination may be considered an effective alternative rather than statistically superior to BAP alone. Statistical analyses confirmed normality, homogeneity of variance, and significant differences among treatments ($p < 0.05$). A hormone-free MS medium (MS0) served as a negative control and failed to induce any shoot buds, confirming the essential role of cytokinins in axillary bud–mediated organogenesis.The present investigation was limited to the shoot induction and multiplication phase of *in vitro* culture. These results demonstrated that the optimized combination of BAP and kinetin enhances direct shoot organogenesis and multiplication in *B. monnieri*. However, it is recommended to examine root induction and acclimatization to establish a complete micropropagation protocol.

Keywords: Bacopa monnieri, Cytokinins, Explant, Kinetin, MS medium, Shoot induction

INTRODUCTION

Brahmi, *Bacopa monnieri* (L.) Wettst. (Scrophulariaceae), is one of the oldest and most powerful brain tonics in the Ayurvedic system from ancient times. It is identified as medhya rasayana in Indian i.e., a drug that is supposed to antagonize the effects of mental stress and improve intelligence and memory function.It is widely distributed in tropical and subtropical regions of Asia, Africa, and America, and has been extensively used in Ayurvedic, Unani, and traditional systems of medicine (Chauhan,1999; Das and Jahan, 2021). It is a creeping, glabrous, succulent herb that roots at nodes, with a habitat that includes wetlands and muddy shores. Flower of brahmi are white or pale-bluish in colour and bloom in axillary and/or solitary arrangement on long slender pedicels (Jaspreet *et al.*,2013).

The plant is pharmacologically significant due to the presence of bioactive saponins, primarily bacosides, which exhibit neuroprotective, antioxidant, anti-inflammatory, and memory-enhancing properties (Singh and Dhawan, 1997; Singh and Singh, 2009; Phrommee *et al.,* 2021). Owing to its cognitive-enhancing potential, *B. monnieri* is often referred to as a “memory tonic” and has been incorporated in various herbal formulations for treating neurological disorders such as Alzheimer’s disease, and epilepsy (Kumar *et al.,* 2022). It was also found to be effective in treatment of anxiety and neurosis also (Vohora *et al.,*1997; Agrawal, 1993).

The increasing global demand for *B. monnieri* in the pharmaceutical and nutraceutical industries has led to overharvesting from natural populations, thereby threatening its availability and genetic diversity. Conventional propagation methods via seeds and stem cuttings are inefficient due to low seed viability, seasonal dependence, and slow growth rates (Khan *et al.,* 2021). In this context, plant tissue culture has emerged as a reliable tool for large-scale propagation, genetic conservation, and secondary metabolite production in such valuable medicinal plants (Ramesh *et al.,* 2023).

Among various tissue culture approaches, in vitro regeneration through shoot induction is highly influenced by plant growth regulators (PGRs), particularly cytokinins such as 6-benzylaminopurine (BAP) and kinetin. Cytokinins regulate cell division, axillary bud proliferation, and shoot differentiation, thereby playing a crucial role in morphogenesis (Singh *et al.,* 2022). Previous studies on *B. monnieri* have demonstrated that BAP enhances shoot multiplication, while kinetin supports elongation and synergizes with BAP to improve regeneration efficiency (Kumar *et al.,* 2022; Meena *et al.,* 2023). However, the response to PGRs is species-specific and concentration-dependent, requiring optimization for efficient micropropagation (Chakraborty *et al.,* 2020).

Despite several reports on tissue culture of *B. monnieri*, comparative analyses of BAP, kinetin, and their combinations on shoot induction remain limited, particularly under controlled in vitro conditions. Optimizing cytokinin concentrations and combinations is essential not only for enhancing shoot multiplication but also for producing genetically uniform, true-to-type plantlets, which are critical for sustainable commercial cultivation and pharmaceutical applications (Bose *et al.,* 2021).

The present study was therefore undertaken to evaluate the effect of BAP, kinetin, and their combinations on direct shoot regeneration through axillary bud activation in *B. monnieri* nodal explants.The study was specifically to optimize cytokinin-mediated shoot induction and multiplication under in vitro conditions, rather than to establish a complete micropropagation protocol that includes rooting and acclimatization.

MATERIALS AND METHODS

Source of explant

The presented study was conducted during July 2023 to January 2024. Specimens of *B. monnieri* were initially collected from and taxonomically authenticated by the Department of Botany, Shri Guru Ram Rai University, Dehradun. Following authentication, the plant material was maintained under controlled conditions in the garden and nursery facilities of School of Agricultural Sciences, Shri Guru Ram Rai University to ensure a continuous supply of healthy stock. The experimental material used in this study was derived from a single healthy mother plant of *B. monnieri*, vegetatively propagated via stem cuttings and maintained under controlled nursery conditions. Thus, all nodal explants employed in the present investigation originated from a single clonal genotype, ensuring genetic uniformity and minimizing variability arising from genotypic differences.For subsequent experimental studies, nodal segments of the plant were carefully excised and utilized as explants for propagation and related investigations at Department of Biotechnology, School of Basic and Applied Sciences, Shri Guru Ram Rai University, Dehradun.

Surface sterilization

The nodal segments were carefully excised from healthy mother plants and initially washed under running tap water to remove adhering dust particles and other superficial debris. To achieve preliminary surface sterilization, the explants were immersed in an aqueous solution of liquid detergent (1–2 drops per 100 mL of water) for approximately 15 minutes, followed by thorough rinsing with distilled water 4–5 times to completely remove detergent residues. Subsequently, the explants were treated with 0.2% (w/v) Bavistin (a systemic fungicide) for 20 minutes to minimize fungal contamination. For final surface sterilization, the explants were treated with 0.1% (w/v) mercuric chloride solution for 2–3 minutes, with intermittent rinsing at each step with sterile double-distilled water to remove traces of the sterilant. Following inoculation on culture media, observations were systematically recorded to assess the percentage of contamination-free cultures after four weeks of incubation.

In vitro establishment of cultures

For each treatment, a total of 30 nodal explants were used per experimental run and distributed across three independent culture vessels (biological replicates), with each vessel containing 10 explants. Each culture vessel represented an independent biological replicate and served as the true experimental unit for statistical analysis (n = 3 per treatment).One nodal explant was inoculated per culture tube to avoid competition and to en-

sure independent axillary bud–mediated shoot regeneration without intervening callus formation. Each culture vessel constituted one independent experimental unit, and the experiment was repeated three times (n = 3 biological replicates per treatment). One nodal explant was inoculated per culture tube to avoid competition and to ensure independent axillary bud–mediated shoot regeneration.

The experiment was conducted in three independent temporal runs. In each run, 30 explants per treatment were distributed across three culture vessels (10 explants per vessel), with each vessel treated as one independent biological replicate. Thus, a total of nine biological replicates (n = 9 vessels per treatment) were included across the three experimental runs. Vessel-wise means from all independent runs were pooled for statistical analysis after confirming consistency across runs.

Explants within the same culture vessel were not treated as independent replicates for statistical analysis, but were used only to calculate mean values per vessel.

Following sterilization, the explants were carefully inoculated onto culture vessels containing full-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962). The medium was fortified with 3% (w/v) sucrose as the primary carbon source to support energy requirements and osmotic balance, and solidified with 0.8% (w/v) agar to provide a semi-solid support for explant growth. To evaluate the influence of plant growth regulators (PGRs) on shoot induction and multiplication, the MS medium was supplemented with varying concentrations of cytokinins. Treatments included medium enriched with 6-benzylaminopurine (BAP) alone at concentrations ranging from 0.5 mg/L to 3.5 mg/L, kinetin alone at concentrations ranging from 0.5 mg/L to 3.0 mg/L, as well as different combinations of BAP (0.5–2.5 mg/L) and kinetin (1.0–3.0 mg/L). These combinations were specifically designed to assess the synergistic or individual effects of the cytokinins on the regenerative response of nodal explants, with the objective of standardizing an efficient protocol for multiple shoot induction. In addition to cytokinin-supplemented treatments, a hormone-free MS medium (MS0) was included as a true negative control to assess the inherent regeneration potential of nodal explants in the absence of plant growth regulators.

Culture medium and conditions

After inoculation, the culture vessels were transferred to a controlled culture room, where environmental parameters were maintained to support optimum growth. The cultures were incubated at 25 ± 2 °C, a temperature considered suitable for most tissue culture experiments, as it mimics *B. monnieri's* natural tropical habitat and promotes active cell division and morphogenesis. A photoperiod of 16 hours of light and 8 hours of dark was provided, simulating long-day conditions, which are known to enhance photosynthetic activity and stimulate shoot induction in many plant species. Illumination was provided by cool-white fluorescent tube lights (40 W), producing an average light intensity of approximately 2500 lux, ensuring uniform light distribution without causing photooxidative stress.

To maintain healthy growth and continuous proliferation, the cultures were sub-cultured at regular intervals of 25–30 days, during which fresh medium was provided and observations on shoot induction were recorded. Sub-culturing not only replenishes nutrients but also reduces the accumulation of toxic metabolites that might inhibit growth.

The pH of the culture medium was adjusted to 5.8 before autoclaving, as this slightly acidic environment has been reported to be optimal for nutrient availability and tissue response in plant tissue culture systems. Deviations from this pH could impair nutrient uptake, reduce the medium's gel strength, or negatively affect enzymatic activities essential for morphogenesis.

Culture duration and observation period

All cultures were maintained for a uniform duration of four (4) weeks following inoculation before quantitative observations were recorded. All quantitative observations were recorded after four weeks of culture. For each treatment, data from individual explants within a culture vessel were first pooled to obtain a single vessel-wise mean, and these vessel-wise means (n = 3) were used as independent biological replicates for all statistical analyses, thereby ensuring accurate estimation of variability and statistical power.Although early axillary bud break was visually observed at different time points depending on treatment (3–5 weeks), all numerical data for percentage response, shoot number, and shoot length were recorded strictly at the end of the fourth week to ensure uniformity and statistical comparability across treatments.

Definitions and measurement of growth parameters

To ensure clarity and reproducibility, the evaluated parameters were defined as follows:

Percentage response (%)

Calculated as the percentage of explants that produced at least one directly regenerated shoot originating from pre-existing axillary buds, relative to the total number of explants inoculated per treatment using the formula:

$$Percentage\ response = \left(\frac{Number\ of\ explants\ showing\ shoot\ induction}{Total\ explants\ inoculated} \right) \times 100$$

Eq.1

Shoot number

Expressed as the average number of shoots produced per responding explant, calculated by dividing the total number of shoots by the number of responding ex-

plants within each treatment.

Shoot length (cm)

Measured as the mean length of the longest shoot per responding explant, recorded from the basal node to the shoot apex using a calibrated ruler.

Statistical analysis

All experiments were conducted in triplicate, and the results are presented as mean ± standard error of the mean (SEM). Data on percentage response, shoot number, and shoot length under different BAP concentrations were statistically analyzed. Percentage data were arcsine-transformed prior to analysis. A one-way Analysis of Variance (ANOVA) was performed to test the effect of hormone concentration, with normality and homogeneity of variance confirmed using the Shapiro–Wilk and Levene's tests, respectively. All statistical analyses were carried out using IBM SPSS Statistics version 26.0, which is widely available and commonly used in academic institutions in India.Statistical analyses were carried out using SPSS, and significance was accepted at $p < 0.05$. Data presented in Tables 1–3 represent pooled mean ± SEM from nine independent biological replicates (three vessels per run × three independent experimental runs; n = 9).

RESULTS

All quantitative data presented in Tables 1-3 represent observations recorded uniformly after four weeks of culture. Nodal explants cultured on hormone-free MS medium (MS0) did not exhibit any shoot bud induction, axillary bud break, or elongation even after four weeks of culture, indicating the absence of spontaneous regeneration under in vitro conditions. All regeneration responses observed in cytokinin-supplemented treatments were characterized by direct shoot organogenesis arising from pre-existing axillary meristems, with no callus-mediated or de novo shoot formation. During axillary bud–mediated direct organogenesis, maximum regeneration responses were observed at 2.5 mg/L BAP alone (Table 1 and Fig.1) and 2.0 mg/L kinetin alone (Table 2and Fig.1), while, the combination of 1.0 mg/L BAP + 1.5 mg/L kinetin produced the highest mean number of shoots per explant; however, it was statistically at par with 0.5 mg/L BAP + 1.0 mg/L kinetin (Table 3 and Fig.1)., where rapid axillary bud break followed by multiple shoot proliferation was recorded. The cytokinins (BAP and Kinetin) facilitate cell division and cell elongation in dose dependent manner. The maximum response was observed with the combination of two hormones; both the hormone response and its concentration were statistically significant ($P<0.05$). The lack of response in MS0 establishes a clear baseline and confirms that all observed regeneration responses in Tables 1–3 were induced exclusively by cytokinin supplementation rather than by endogenous hormonal activity of the explants.

The influence of different concentrations of BAP (0.5–3.5 mg/L) on shoot induction is presented in Table 1. No response was observed at 0.5 mg/L, while a low response (16.6%) was recorded at 1.0 mg/L with an average of 2.0 ± 1.1 shoots per explant and 1.1 ± 1.3 cm shoot length. The response improved progressively with increasing concentration, and the maximum shoot induction was observed at 2.5 mg/L BAP with 58.3% response, 7.0 ± 0.3 shoots, and 2.2 ± 0.9 cm shoot length. Further increase in BAP concentration (3.5 mg/L) reduced the response (33.3%), number of shoots (4.0 ± 0.3), and shoot elongation (1.4 ± 1.1 cm). No response was observed at 0.5 mg/L BAP, which was consistent with the absence of regeneration on MS0, while the maximum response (58.3%) was recorded at

Table 1. Effect of BAP on axillary bud-mediated direct shoot organogenesis in *Bacopa Monnieri*

S. No.	BAP (mg L^{-1})	Explants responding (%)	Shoots per responding explant (Mean ± SEM)	Shoot length (cm) (Mean ± SEM)
1	0.0 (MS_0)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	1.0	16.6 ± 0.3	2.0 ± 1.1	1.1 ± 1.3
4	1.5	33.3 ± 1.2	4.0 ± 1.9	1.5 ± 1.2
5	2.5	58.3 ± 0.8	7.0 ± 0.3	2.2 ± 0.9
6	3.5	33.3 ± 0.2	4.0 ± 0.3	1.4 ± 1.1
One-way ANOVA summary				
Parameter		**df (Between, Within)**	**F-value**	**Significance**
Explants responding (%)		5, 12	701.25	*** ($p < 0.001$)
Shoots per responding explant		5, 12	7.22	** ($p < 0.01$)
Shoot length (cm)		5, 12	0.61	NS ($p > 0.05$)

MS_0 = hormone-free Murashige and Skoog medium (true negative control);Values represent mean ± SEM;Data are statistically significant at $P < 0.05$

Table 2. Effect of kinetin on axillary bud mediated direct shoot organogenesis in *Bacopa monnieri*

S. No.	Kinetin (mg L^{-1})	Explants responding (%)	Shoots per responding explant (Mean ± SEM)	Shoot length (cm) (Mean ± SEM)
1	0.0 (MS_0)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	1.0	25.0 ± 1.2	3.0 ± 0.8	1.2 ± 0.4
4	1.5	33.3 ± 1.9	4.0 ± 1.4	1.6 ± 0.2
5	2.0	50.0 ± 1.1	6.0 ± 1.8	2.1 ± 1.4
6	3.0	33.3 ± 0.9	4.0 ± 1.5	1.4 ± 1.1
One-Way ANOVA summary				
Parameter		**F-value**	**Significance**	**CD (0.05)**
Explants responding (%)		339.60	***	4.86
Shoots per explant		4.28	*	3.25
Shoot length (cm)		1.34	NS	—

MS_0 confirms the absence of spontaneous regeneration under in vitro conditions;Values represent mean ± SEM; significance at P < 0.05;Optimum kinetin concentration was 2.0 mg L□¹, beyond which inhibitory effects were observed

2.5 mg/L BAP.

The data in Table 2 show the effects of kinetin concentrations (0.5–3.0 mg/L). Explants cultured on medium supplemented with 0.5 mg/L kinetin showed no morphogenetic response. At 1.0 mg/L, 25.0% of explants responded with 3.0 ± 0.8 shoots per explant and 1.2 ± 0.4 cm shoot length. The best response was obtained at 2.0 mg/L kinetin, where 50.0% of explants regenerated with an average of 6.0 ± 1.8 shoots and 2.1 ± 1.4 cm shoot length. A further increase to 3.0 mg/L caused a decline in regeneration response (33.3%) and average shoot number (4.0 ± 1.5). Similarly, explants cultured on MS0 with 0.5 mg/L kinetin failed to show morphogenetic responses, whereas those cultured with 2.0 mg/L kinetin showed optimal regeneration.

The enhanced morphogenic response of BAP and kinetin on shoot induction is summarized in Table 3.Combined treatment significantly improved shoot induction compared to individual PGRs. The highest response (66.6%) was achieved at 1.0 mg/L BAP + 1.5 mg/L kinetin, producing an average of 8.0 ± 0.9 shoots per explant and 2.6 ± 1.9 cm shoot length. Other combinations showed comparatively lower responses, with 0.5 + 1.0 mg/L inducing 50.0% response and 6.0 ± 1.1 shoots per explant. Increasing the concentrations beyond the optimum resulted in a gradual decline in shoot induction frequency, number, and length. The improved effect of BAP and kinetin was evident, with the highest regeneration frequency (66.6%) at 1.0 mg/L BAP + 1.5 mg/L kinetin. Compared to the absolute zero response on MS0, this represents a substantial cytokinin-driven enhancement of morphogenesis.

For BAP treatments, the Shapiro–Wilk test values for % response (W = 0.969, p = 0.872), shoot number (W = 0.969, p = 0.872), and shoot length (W = 0.945, p = 0.699) indicated that the data did not significantly deviate from normal distribution (p > 0.05). Similarly, Levene's test confirmed homogeneity of variance

Table 3. Combined effect of BAP and kinetin on axillary bud mediated direct shoot organogenesis in *Bacopa monnieri*

S. No.	BAP + kinetin (mg L^{-1})	Explants responding (%)	Shoots per responding explant (Mean ± SEM)	Shoot length (cm) (Mean ± SEM)
1	0.0 +0.0 (MS_0)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.5 + 1.0	50.0 ± 1.8	6.0 ± 1.1	2.0 ± 2.1
3	1.0 + 1.5	66.6 ± 1.4	8.0 ± 0.9	2.6 ± 1.9
4	1.5 + 2.0	50.0 ± 1.4	6.0 ± 0.8	2.1 ± 1.3
5	2.0 + 2.5	41.6 ± 0.3	5.0 ± 1.1	1.7 ± 1.2
6	2.5 + 3.0	33.3 ± 0.9	4.0 ± 1.2	1.5 ± 0.8
One-way ANOVA summary				
Parameter		**F-value**	**Significance**	**CD (0.05)**
Explants responding (%)		530.43	***	4.32
Shoots per explant		15.28	**	2.13
Shoot length (cm)		0.81	NS	—

Values represent mean ± SEM & data significant at P < 0.05; Combination 1.0 mg L□¹ BAP + 1.5 mg L□¹ kinetin showed a enhanced effect, producing the highest statistically significant regeneration frequency, shoot number, and shoot length; Decline at higher concentrations indicates cytokinin dose-dependent inhibition

Table 4. Shapiro–Wilk (normality) and Levene's (homogeneity of variance) tests response of different BAP treatments on in vitro axillary bud–mediated direct shoot organogenesis in *Bacopa monnieri*

Treatment	Parameter	Shapiro–W	Shapiro p	Levene–F	Levene p
BAP	% Response	0.969	0.872	0.845	0.410
BAP	Shoot number	0.969	0.872	0.844	0.410
BAP	Shoot length	0.945	0.699	0.669	0.459
Kinetin	% Response	0.932	0.607	0.583	0.488
Kinetin	Shoot number	0.932	0.607	0.583	0.488
Kinetin	Shoot length	0.980	0.917	0.500	0.512
BAP+Kinetin	% Response	0.973	0.884	0.750	0.442
BAP+Kinetin	Shoot number	0.963	0.828	0.667	0.461
BAP+Kinetin	Shoot length	0.958	0.799	0.600	0.478

% Response: Percentage of explants showing shoot induction;Shapiro–W: Shapiro–Wilk test statistic for normality;Shapiro p: p-value of Shapiro–Wilk normality test; Levene–F: Levene's test statistic for homogeneity of variance; Levene p: p-value of Levene's test

across BAP concentrations with *p* values greater than 0.05 (Table 4). The Shapiro–Wilk test did not indicate a significant deviation from normality; however, given the moderate sample size, the results were interpreted cautiously.

In the case of Kinetin treatments, Shapiro–Wilk values for % response (W = 0.932, p = 0.607), shoot number (W = 0.932, p = 0.607), and shoot length (W = 0.980, p = 0.917) also confirmed normal distribution. The Levene's test results for the same parameters (% response: F = 0.583, p = 0.488; shoot number: F = 0.583, p = 0.488; shoot length: F = 0.500, p = 0.512) indicated that group variances were statistically homogeneous.

For the combined BAP + Kinetin treatments, all parameters (% response, shoot number, and shoot length) exhibited Shapiro–Wilk *p*-values greater than 0.05, confirming normality (e.g., % response: W = 0.973, p = 0.884). Similarly, Levene's test showed no evidence of unequal variances across treatment groups (e.g., shoot number: F = 0.667, p = 0.461).

Overall, both normality and homogeneity assumptions were satisfied across all treatments and measured parameters. These results confirmed that the datasets met the prerequisites for parametric testing and could be appropriately applied to evaluate the effects of different concentrations of BAP, Kinetin, and their combination on in vitro shoot bud induction in *B. monnieri*.

The present results revealed that the concentration of the plant growth regulator significantly influenced shoot bud induction in *B. monnieri*. Among the individual treatments, 2.5 mg/L BAP resulted in the highest shoot induction response (58.3%), shoot number (7.0), and shoot length (2.2 cm), which were statistically superior to lower and higher concentrations. Similarly, 2.0 mg/L kinetin was the most effective treatment, producing significantly greater responses than the other concentrations tested.

When BAP and Kinetin were used in combination, the treatment with 1.0 mg/L BAP + 1.5 mg/L Kinetin showed the highest response (66.6%), with the greatest number of shoots (8.0) and the longest shoot length (2.6 cm).The combination of 1.0 mg/L BAP + 1.5 mg/L kinetin produced the highest numerical regeneration response (66.6%) and shoot number (8.0 ± 0.9). However, Tukey's HSD post hoc comparison revealed that shoot number did not differ significantly from the best individual BAP treatment (7.0 ± 0.3 at 2.5 mg/L BAP; p > 0.05). Therefore, while the combination showed a numerically higher response, it cannot be considered statistically superior to BAP alone.

Thus, throughout this study, the term "optimum" refers to the concentration that produces the highest statistically significant regeneration response, shoot number, and shoot length, as supported by the quantitative data presented in Tables 1-3.

DISCUSSION

The present study demonstrates that BAP, kinetin, and their combinations play crucial roles in regulating axillary bud–mediated direct shoot organogenesis in *B. monnieri*. The complete absence of regeneration on hormone-free MS medium (MS0) confirms that nodal explants lack inherent regenerative capacity under in vitro conditions and require exogenous cytokinin supplementation for activation and proliferation of pre-existing axillary meristems, rather than de novo shoot formation.

Among individual treatments, BAP at 2.5 mg/L was most effective, while kinetin at 2.0 mg/L also supported shoot induction but with a slightly lower response (Table 1 and Fig.1). Although the combined treatment yielded the highest shoot number, statistical analysis showed no significant difference from the optimal BAP treatment alone (p > 0.05). Therefore, the interaction between BAP and kinetin may represent an effective

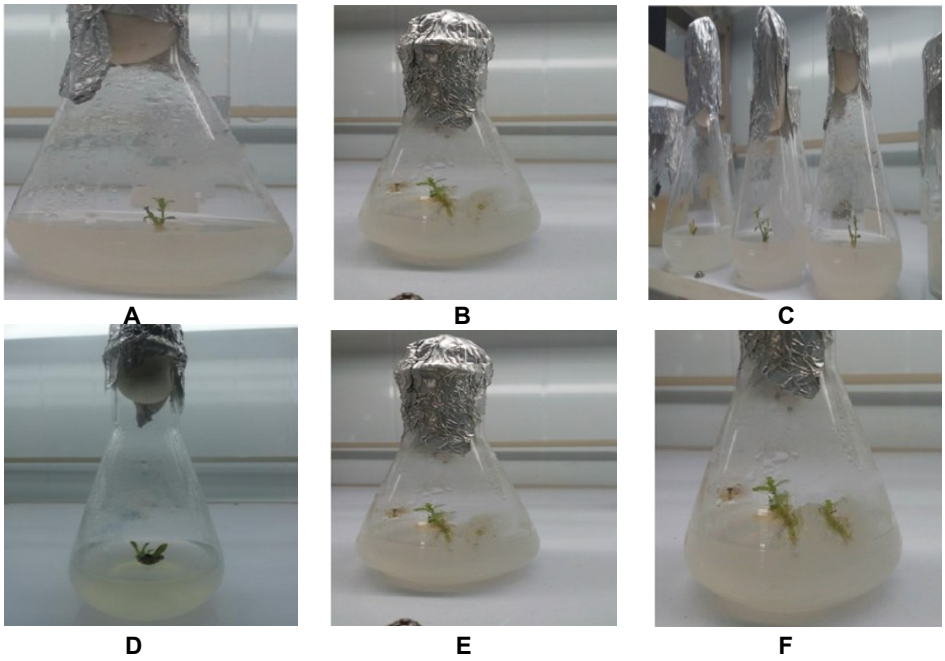

Fig. 1. *(A–F) Axillary bud–mediated direct shoot organogenesis in bacopa monnieri ;A. Bud break from nodal explants;B. Initiation of multiple shoots;C. Establishment of in vitro culture;D. Shoot bud induction on MS + 2.0 mg/L BAP (representative response observed by 4 weeks);E. Shoot bud induction on MS + 2.5 mg/L kinetin (representative response observed by 4 weeks);F. Enhanced axillary shoot proliferation on MS + 1.0 mg/L BAP + 1.5 mg/L kinetin (representative response observed by 4 weeks)*

hormonal balance rather than a statistically superior enhancement effectThe inclusion of MS0 as a true negative control strengthens the experimental design by providing a definitive baseline, thereby allowing absolute quantification of cytokinin effects rather than relative comparisons alone.

The superiority of BAP over kinetin in shoot induction observed in this study aligns with earlier findings in *B. monnieri* and other medicinal plants (Patel *et al.,* 2021; Kumar *et al*., 2022). BAP is widely reported to be more effective than kinetin due to its higher stability and stronger interaction with cytokinin receptors, thereby promoting cell division and axillary bud proliferation (Zhang *et al.,* 2020). Similarly, Das and Jahan (2021) reported that 2.5 mg/L BAP produced the highest number of shoots in *B. monnieri* cultures, corroborating the present findings. The enhanced shoot proliferation observed in BAP-treated cultures may be hypothetically explained, based on previous reports, by BAP's relatively greater stability and reported efficacy in cytokinin signalling pathways. Similarly, earlier studies have suggested that elevated cytokinin levels can suppress apical dominance, thereby promoting axillary bud proliferation. However, these mechanistic interpretations are derived from existing literature and were not directly examined in the present study.

The role of kinetin in shoot induction, although less pronounced than that of BAP, was evident at a moderate concentration (2.0 mg/L) (Table 2 and Fig.1). Excess kinetin reduced shoot induction efficiency, likely due to supra-optimal cytokinin levels, leading to physiological stress or callus formation rather than organized shoot morphogenesis (Mahato *et al*., 2000; Khan *et al.,* 2021; Ramesh *et al.,* 2023).

Interestingly, the combined application of BAP and kinetin exhibited a clear enhancement effect, particularly at 1.0 + 1.5 mg/L (Table 3 and Fig.1). Cytokinin interactions are known to enhance morphogenesis through complementary signalling pathways, resulting in increased cell proliferation and bud differentiation (Sundriyal *et al*., 2013; Singh *et al.,* 2022). Similar effects of BAP and kinetin on shoot multiplication have been reported in *Centella asiatica* (Bose *et al.,* 2021) and *Withania somnifera* (Meena *et al.,* 2023).

Shoot length was also influenced by PGRs, with moderate cytokinin concentrations promoting elongation while higher doses reduced shoot length. This reduction may be attributed to cytokinin-induced suppression of apical dominance at higher concentrations, which limits elongation (Tripathi and Tripathi, 2003; Chakraborty *et al.,* 2020). The maximum shoot length observed in the present study (2.6 ± 1.9 cm) under BAP + kinetin treatment is comparable to earlier reports in *B. monnieri*, highlighting the importance of optimizing cytokinin balance for both multiplication and elongation.

Among individual cytokinins, 2.5 mg/L BAP and 2.0 mg/L kinetin consistently produced the maximum regeneration responses, confirming these concentrations as optimal for individual PGR-mediated axillary shoot regeneration in *B. monnieri*.While the present study suc-

cessfully optimized cytokinin-mediated shoot induction and multiplication in *B. monnieri*, it is important to note that the protocol currently addresses only the shoot regeneration phase of micropropagation. Root induction of regenerated shoots and their subsequent acclimatization under ex vitro conditions were not examined in this study. These stages are critical for the survival, establishment, and large-scale deployment of in vitro–raised plantlets. Therefore, the findings can be considered as a foundational step toward the development of a complete micropropagation protocol. Although the increase in shoot number under the combined cytokinin treatment was not very large, even small improvements in multiplication rate can make a meaningful difference in large-scale micropropagation. Over repeated subcultures, a slight increase per cycle can significantly improve overall plant production while saving time and cost. In this study, the combination of 1.0 mg/L BAP and 1.5 mg/L kinetin proved to be the most effective for enhancing shoot regeneration, multiplication, and elongation compared to using either cytokinin alone.

It is important to note that the study focused only on shoot multiplication. Rooting and acclimatization were not included, so the results represent optimization of the shoot-induction stage rather than a complete micropropagation protocol.All explants were taken from a single clonal mother plant, meaning the differences observed are mainly due to hormonal treatments. However, prolonged exposure to BAP can sometimes lead to somaclonal variation or hyperhydricity. In this study, shoots developed directly from nodal explants with minimal callus formation, which lowers the risk of genetic variation. Still, molecular marker analysis would be needed to confirm clonal uniformity before large-scale commercial use. High cytokinin levels during multiplication may also affect later rooting due to hormonal carryover. Therefore, transferring shoots to a cytokinin-free medium or an auxin-supplemented rooting medium may be necessary, and this should be examined in future studies.

Finally, since only a single B. monnieri genotype was used, the results should be validated across different accessions. The effect of cytokinin treatments on bacoside content was also not assessed and deserves further phytochemical investigation. Validation of genetic fidelity in regenerated plantlets using molecular markers is also recommended as a future step to confirm their suitability for sustainable commercial propagation and conservation.

Conclusion

BAP at 2.5 mg/L and kinetin at 2.0 mg/L were identified as optimal individual treatments, while the combination (1.0 mg/L BAP + 1.5 mg/L kinetin) produced the highest numerical regeneration efficiency in *B. monnieri*; however, it was not statistically different from the optimal BAP treatment alone. Therefore, both treatments may be considered effective for cytokinin-mediated shoot multiplication. The results demonstrated that cytokinins promote axillary bud–mediated shoot induction, multiplication, and elongation in a dose-dependent manner. No shoot regeneration occurred on hormone-free MS medium, confirming the essential role of cytokinins in direct shoot organogenesis. This study establishes an optimized cytokinin-based system for *in vitro* shoot induction and multiplication of *B. monnieri* using nodal explants. However, rooting, acclimatization, and field establishment were not investigated in the present study and must be addressed to develop a complete micropropagation protocol. However, further work on rooting, acclimatization, and secondary metabolite profiling is required to develop a complete, commercially viable protocol suitable for large-scale propagation, conservation, and medicinal use of this important species.

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Conflict of interest

The authors declare that they have no conflict of interest.

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