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OPTIMIZATION OF 6-BENZYLAMINOPURINE CONCENTRATION FOR THE MICROPROPAGATION OF BAMBOO (*Guadua angustifolia* Kunth) USING THE RESPONSE SURFACE METHODOLOGY

SUMMARY

In vitro micropropagation of *Guadua angustifolia* constitutes a fundamental biotechnological tool for the large-scale production of genetically uniform, pathogen-free, and continuously available plant material. In this context, the objective of this study was to optimize the concentration of 6-benzylaminopurine (6-BAP) for the in vitro micropropagation of *G. angustifolia* using Response Surface Methodology (RSM). Explants were disinfected through sequential rinses, immersion in detergent (20 g L^{-1}), Hachero® fungicide (3 ml L^{-1}), 70% ethanol, and 1% sodium hypochlorite, with constant agitation between each stage. A single-factor response surface design was implemented, evaluating 6-BAP concentrations ranging from 0 to 5 mg L^{-1} in the micropropagation of *G. angustifolia*. The disinfection protocol showed 95% explant survival, only 5% contamination, and no necrosis. Results from the 23 experimental runs of the single-factor RSM design optimized the 6-BAP concentration for micropropagation. The obtained quadratic model was highly significant ($p<0.0001$; $R^2=0.9735$), demonstrating a strong positive correlation ($r=0.925$) between 6-BAP concentration and the number of shoots. The optimal concentration was determined to be 4.83 mg L^{-1}, producing up to an average of 3.57 shoots per explant, confirming the necessity of cytokinins to maximize in vitro multiplication. These results establish an efficient protocol for the

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micropropagation of *G. angustifolia*, facilitating its large-scale and controlled production through standardized biotechnological methodologies.

Keywords: bamboo, cytokinins, micropropagation, biotechnology

INTRODUCTION

In vitro micropropagation has become a key biotechnological methodology for large-scale plant production, enabling the acquisition of genetically homogeneous, pathogen-free plant material that can be obtained year-round (Farkas *et al.,* 2025). For species of significant ecological and economic importance, such as Guadua angustifolia, this technique represents a strategic alternative to the limitations associated with traditional propagation methods, which are often characterized by low rooting efficiency, seasonal constraints, and issues related to disease transmission (Tejada-Alvarado *et al*., 2022).

G. angustifolia is a native bamboo species from the tropical regions of the Americas. Its importance lies in its wide range of applications, from sustainable construction, furniture manufacturing, and handicrafts, to its crucial role in ecological restoration, soil recovery, and watershed protection (Muñoz-López *et al.,* 2020). Moreover, this species exhibits rapid growth and a remarkable carbon sequestration capacity (Ureta-Leones *et al.,* 2024), establishing it as a key resource within climate change mitigation strategies and the sustainable development of tropical ecosystems (Villalba Malaver *et al.*, 2025). However, the increasing demand for this species for both productive and environmental purposes contrasts with the lack of efficient propagation systems, limiting its large-scale utilization and conservation.

Although the in vitro propagation of *G. angustifolia* has shown promising results in experimental studies, challenges remain regarding multiplication efficiency and morphogenetic response (Nogueira, Gomes, Everson, *et al.*, 2019). These aspects are closely related to the hormonal formulation of the culture medium (Tejada-Alvarado *et al.,* 2022). Among the plant growth regulators, 6-benzylaminopurine (6-BAP), a synthetic cytokinin, is widely used for its ability to induce shoot proliferation and activate axillary meristems (Gutiérrez *et al.,* 2016). However, its effectiveness depends on several factors, including concentration, explant type, species, and culture conditions, making its specific optimization necessary.

In this context, RSM emerges as an effective statistical tool for modeling and optimizing complex biotechnological processes, allowing for a reduction in the number of experimental trials while maximizing the information obtained (Arteaga-Crespo *et al.,* 2020). When applied to micropropagation studies, RSM enables the establishment of functional relationships between hormonal concentrations and response variables (such as number of shoots, length, vigor, or rooting rate), facilitating the identification of optimal experimental conditions (Khajehyar *et al.,* 2024).

The objective of this study was to optimize the concentration of 6-benzylaminopurine for the in vitro micropropagation of *G. angustifolia* using

RSM, contributing to the development of efficient protocols that maximize multiplication rates, minimize morphogenetic abnormalities, and establish the basis for large-scale production of this species under controlled conditions, thereby strengthening propagation strategies for tropical bamboos.

MATERIAL AND METHODS

Study area

The study was conducted in the Plant Biotechnology Laboratory of the Centro Experimental de Investigación y Producción Amazónica (CEIPA) at the Universidad Estatal Amazónica, located in Arosemena Tola, Napo Province, Ecuador (geographical coordinates: 1°14'17.6"S, 77°53'07.7"W). This center covers an area of 2,848 hectares, of which approximately 2,500 correspond to native forest. The region is characterized by a typically warm and humid climate, with average annual temperatures ranging from 24 to 25 °C, an average annual precipitation of about 4,000 mm, altitudes between 580 and 990 m above sea level, and a relative humidity close to 80% (Ureta-Leones *et al.*, 2024).

Plant material

The plant material was obtained from *G. angustifolia* plants established in the plots of the CEIPA Forestry Program, which were one year old and exhibited adequate morphological and physiological development. Prior to explant collection, donor plants of *G. angustifolia* were subjected to a preventive antifungal treatment through foliar spraying with the fungicide Hachero® ($CuSO_4 \cdot 5H_2O$, 247 g L^{-1}) at a working concentration of 3 ml L^{-1}. The pretreatment was applied every two days for a period of six days before collecting the plant material. Primary branches were selected and collected, and explants were obtained (1.5 cm at both ends of the node). Each explant contained an axillary bud protected by a sheath leaf, which exhibited a yellowish-green coloration (Tejada-Alvarado *et al.*, 2022). The explants were transported in a glass container with a citric acid solution (100 mg L^{-1} distilled water) for 30 minutes to prevent phenolic oxidation (Suwal *et al.*, 2020).

Disinfection of plant material

In the laboratory, the explants were initially rinsed with distilled water, and the sheath leaf was removed. Subsequently, a second rinse with distilled water was performed, followed by immersion in a commercial detergent solution at a concentration of 20 g L^{-1} for 10 minutes. Three consecutive rinses with sterile distilled water were then carried out. Under aseptic conditions, the explants were treated with the fungicide Hachero® at a working concentration of 3 ml L^{-1}, followed by three additional rinses with sterile distilled water. The explants were then immersed in 70% ethanol for 1 minute, rinsed three times, and finally treated with a 1% sodium hypochlorite solution for 10 minutes. Throughout each stage of the disinfection process, the explants were kept under constant agitation to ensure the effective action of the disinfecting agents (Correa *et al.*, 2014).

Experimental design and data analysis

The experimental design was based on the application of a single-factor RSM, using the software Design Expert, version 13 (Minneapolis, MN, USA),

following the methodological approach proposed by Arteaga-Crespo *et al.*, (2021). The objective was to determine the optimal concentration of 6-BAP for the in vitro micropropagation of *G. angustifolia*. The range of treatments included a control (0 mg L^{-1}) and a maximum concentration of 5 mg L^{-1}, established based on values reported in previous studies (Gutiérrez *et al.*, 2016; Jiménez *et al.*, 2006; Leão *et al.*, 2020). All treatments were established on Murashige and Skoog basal medium (MS; 4.43 g L^{-1}), supplemented with 20 g L^{-1} sucrose as a carbon source and solidified with 8 g L^{-1} agar-agar as a gelling agent (Murashige and Skoog, 1962). The program generated a set of concentrations to be evaluated under the RSM design (Table 1), implemented in three replicates, each consisting of a block of 10 explants.

Table 1. Single-factor RSM experimental design: 6-BAP concentrations and observed and predicted response values

Experiment	6-BAP (mg L^{-1})	Number of Shoots	
		Experimental Value	Predicted Value
1	0.00	0.33	0.19
2	0.00	0.00	0.19
3	2.50	2.67	2.78
4	1.67	2.33	2.12
5	2.50	3.00	2.78
6	2.50	2.67	2.78
7	2.50	2.67	2.78
8	3.75	3.67	3.40
9	0.00	0.33	0.19
10	0.00	0.00	0.19
11	2.50	2.67	2.78
12	2.50	2.67	2.78
13	5.00	3.33	3.57
14	1.25	1.67	1.71
15	2.50	2.33	2.78
16	0.00	0.33	0.19
17	2.50	3.00	2.78
18	5.00	3.33	3.57
19	2.50	2.67	2.78
20	5.00	3.67	3.57
21	5.00	3.67	3.57
22	3.33	3.67	3.25
23	5.00	3.67	3.57

The response variable analyzed was the number of shoots per explant, considered a direct indicator of the efficiency of the in vitro multiplication process. An analysis of variance (ANOVA) was performed to estimate the influence of 6-BAP concentration as an independent variable ($p<0.05$). To determine the optimal concentration of 6-BAP, the quadratic model was simplified to a second-order regression (Equation 1):

$$y=\beta_0+\beta_1 x+\beta_{11}x^2 \quad (1)$$

Where β_0 corresponds to the intercept, β_1 represents the linear effect, and β_{11} denotes the quadratic effect. The optimal concentration of 6-BAP was determined by differentiating the regression function with respect to the independent variable and solving for the value of x that maximizes the response. This procedure was further validated through the optimization functions provided by the statistical software, ensuring the robustness and reproducibility of the result. Model adequacy was determined through the coefficient of determination (R^2) and statistical significance (p). Additionally, model quality and reliability were assessed using the Adequate Precision (Adeq. Precision) value, considering a threshold greater than 4 as indicative of sufficient predictive capability.

RESULTS AND DISCUSSION

Survival and Asepsis of Explants

The disinfection protocol employed proved to be highly effective, achieving a 95% survival rate of explants, while contamination incidence was limited to 5%, with no cases of necrosis recorded.

Table 2. Effectiveness of the disinfection protocol applied to *G. angustifolia* explants

Indicators	Average (%)	Description
Contamination	5	Explants that exhibited microbial growth after establishment.
Necrosis	0	Explants that showed irreversible tissue damage following disinfection.
Survival	95	Viable and contamination-free explants suitable for *in vitro* culture.

Figure 1. In vitro propagation of *G. angustifolia*: a) donor plant of explants, b) middle stem segment showing the cauline leaf, c) axillary buds in the internode, d) shoot proliferation, and e) shoot growth under in vitro culture conditions

These results confirm the efficacy of the procedure in obtaining viable and contaminant-free plant material for subsequent in vitro establishment (Table 2, Figure 1).

Optimization of 6-Benzylaminopurine Concentration

Based on the one-factor RSM design, 23 experimental runs were conducted to determine the optimal concentration of 6-BAP for the in vitro micropropagation of *G. angustifolia*. Table 1 presents the experimental and predicted values used in the development of the statistical model. The results showed a response range between 0 and 3.67 average shoots per explant across the different evaluated 6-BAP concentrations.

Figure 2a illustrates a positive correlation between 6-BAP concentration and the number of shoots produced in *G. angustifolia*. As concentration increased, the number of shoots also rose until reaching a maximum response level. The linear correlation coefficient (r=0.925) indicated that more than 92% of the variation in shoot number was dependent on 6-BAP concentration. Figure 2b shows the normal probability plot of the residuals, where the data points are distributed closely along the reference line. This suggests that the model errors reasonably follow a normal distribution, supporting the statistical validity of the analysis and the reliability of the predictions.

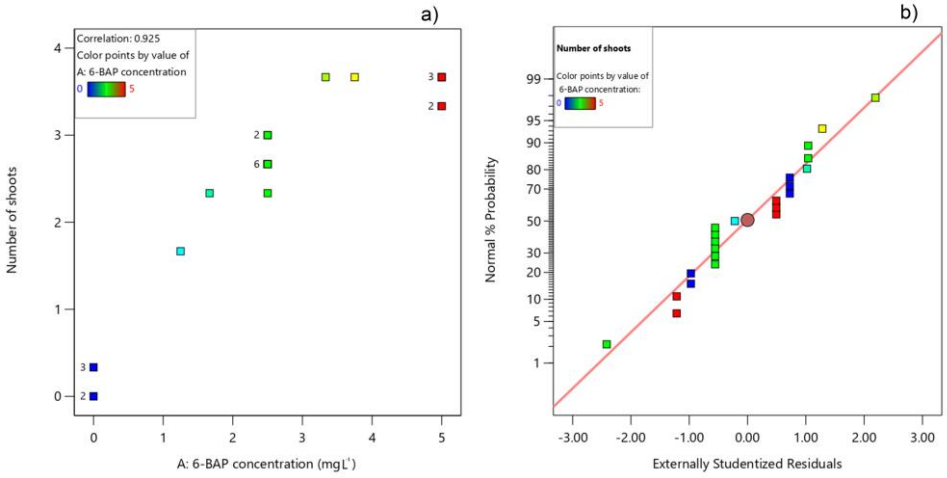

Figure 2. Diagnostic of experimental values: a) Relationship between 6-BAP concentration and number of shoots; b) Normality of residuals.

The analysis of variance (ANOVA) of the quadratic model applied to the response variable "Number of shoots" (Table 3) revealed a highly significant effect of 6-BAP concentration on shoot induction in *G. angustifolia* ($F=367.92$; $p<0.0001$). Both the linear (A) and quadratic (A^2) terms were significant ($p<0.0001$), confirming the direct and non-linear influence of the concentration on the morphogenic response. The model exhibited a high coefficient of determination ($R^2=0.9735$), with adjusted ($R^2adj=0.9709$) and predicted

(R^2pred=0.9660) values in close agreement, indicating a robust and reliable fit of the equation to the experimental data set.

The lack of fit was not significant (p=0.0893), supporting the validity of the model in describing the relationship between 6-BAP concentrations and the number of shoots. Moreover, the adequate precision value (43.01) largely exceeded the recommended threshold (≥4), confirming that the model possesses sufficient precision for navigating the design space.

Table 3. Analysis of variance (ANOVA) of the quadratic model

	Sum of Squares	**df**	**Mean Square**	**F-value**	**p-value**
Quadratic model	34.81	2	17.41	367.92	< 0.0001
A- Concentration	30.59	1	30.59	646.64	< 0.0001
A^2	4.22	1	4.22	89.21	< 0.0001
Residual	0.9462	20	0.0473		
Lack of Fit	0.3585	4	0.0896	2.44	0.0893
Pure Error	0.5877	16	0.0367		
Corrected total	35.76	22			
Std. Dev	0.2175		R^2	0.9735	
Mean	2.36		Adjusted R^2	0.9709	
Coefficient of variation	9.21		Predicted R^2	0.9660	
			Adeq Precision	43.0052	

In Figure 3a, a close fit between the experimental values and the model can be observed, with the points distributed around the equality line (y=x). This demonstrates that the applied quadratic model accurately predicts the observed response, reflecting a low level of error and confirming the statistical robustness of the fit.

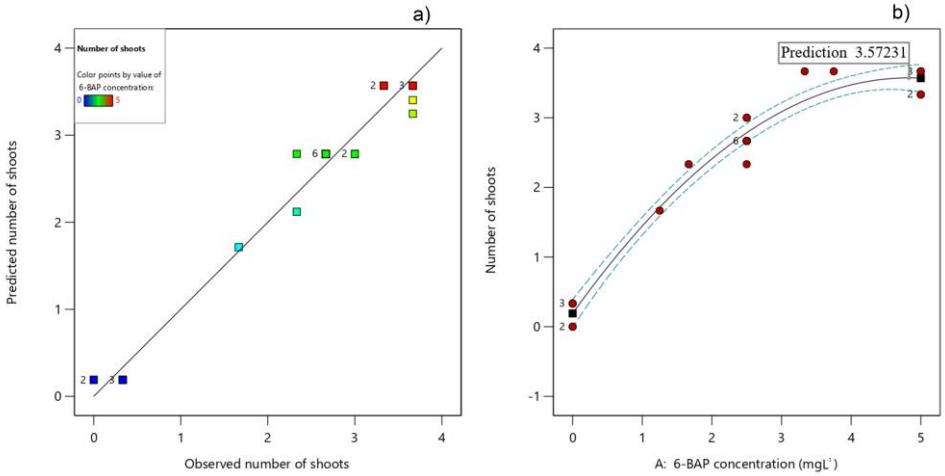

Figure 3. Validation of the quadratic predictive model: (a) Experimental values vs. predicted values; (b) Prediction curve of shoot number.

Finally, Figure 3b shows the quadratic response trend, where the number of shoots increases with the rise in 6-BAP concentration, reaching a predicted maximum value of approximately 3.57 shoots per explant at intermediate concentrations. At higher doses, the response tends to stabilize or slightly decline, confirming a nonlinear hormonal effect and the necessity of identifying an optimal concentration.

Mathematical Model for Estimating Shoots Induced by 6-BAP

Based on the data obtained through the Response Surface Methodology (RSM), the final equation was established in terms of real factors (Equation 2), allowing the estimation of the number of shoots (B) as a function of 6-BAP concentration (A). The model revealed that the number of shoots responded positively to increasing 6-BAP concentrations; however, at higher levels, a decreasing effect was observed, as reflected in the significance of the quadratic term.

$$B=0.190009+(1.23378*A)-(0.144826*A^2) \quad (2)$$

On average, the highest response was achieved at a concentration of 4.83 mg L^{-1} of 6-BAP, whereas the control treatment (0 mg L^{-1}) exhibited the lowest values. These results confirm the importance of supplementing the culture medium with cytokinins to optimize the in vitro multiplication of *G. angustifolia*.

DISCUSSION

The disinfection protocol implemented in this study for *G. angustifolia* proved to be highly efficient, achieving a 95% survival rate of explants, with only 5% contamination and no occurrence of necrosis. This level of effectiveness is consistent with the findings reported by Jiménez *et al.*, (2006), who, by employing alkaline detergent, fungicides, and 1.5% NaOCl for 10 minutes, achieved satisfactory contaminant control in the same species. These authors further enhanced their protocol by incorporating Plant Preservative Mixture® (PPM) into the culture medium, which promoted successful establishment and shoot proliferation. Similar results were reported by Leão *et al.*, (2020), who confirmed that PPM™ effectively reduced bacterial and fungal contamination during bamboo micropropagation. In this context, the strategy used in the present study; combining detergent, copper(II)sulfate pentahydrate, ethanol, and sodium hypochlorite, along with continuous agitation-ensured efficient decontamination without compromising tissue viability, a crucial aspect for successful in vitro establishment.

Beyond contamination control, the primary focus of this research was the optimization of 6-BAP concentration for shoot induction in *G. angustifolia*. The application of the RSM enabled a robust statistical analysis, revealing an outstanding model fit (R^2=0.9735; adjusted R^2=0.9709; predicted R^2=0.9660), with an Adeq. Precision value (43.01) far exceeding the recommended threshold

(≥4). These indicators confirmed both the reliability and predictive capacity of the model. The normal distribution of residuals and the non-significant lack of fit (p=0.0893) further validated the statistical soundness of the design, demonstrating the utility of RSM as a tool to optimize micropropagation protocols for ecologically and economically valuable species (Arteaga-Crespo *et al.*, 2021).

Experimental results revealed a positive correlation between 6-BAP concentration and the number of shoots, reaching a maximum of approximately 3.57 shoots per explant, followed by a slight decline at higher doses. Apical dominance release induced by cytokinins represents a fundamental physiological mechanism driving this proliferation in bamboos. By counteracting the inhibitory effect of auxin gradients from the apical meristem, cytokinins promote axillary bud activation and stimulate cell division in lateral meristems. This hormonal balance explains the quadratic response observed, where intermediate concentrations of 6-BAP maximize shoot induction, while higher levels reduce efficiency due to phytotoxicity or hormonal imbalance. Such quadratic responses are characteristic of cytokinin activity, with stimulatory effects peaking at intermediate doses and declining at elevated levels. These mechanisms have been widely documented in woody species and bamboos, confirming that cytokinin-mediated apical dominance release is central to optimizing micropropagation protocols (Cline, 1997; García-Ramírez, 2023; Nogueira, Gomes and Scherwinski-Pereira, 2019).

At the optimal concentration of 4.83 mg L^{-1}, regenerated shoots exhibited normal morphology, adequate length, and vigor, with no signs of hyperhydricity or structural abnormalities. This observation confirms that the identified dose not only maximizes shoot induction but also preserves the morphological quality of explants, which is essential for ensuring the efficiency and stability of micropropagation protocols.

Prolonged exposure to elevated concentrations of BAP may pose significant risks in bamboo micropropagation, including hyperhydricity, somaclonal variation, and abnormal morphologies. These effects are associated with hormonal imbalance and physiological stress, which compromise genetic stability and the quality of regenerated plantlets. Previous studies have reported that supra-optimal 6-BAP levels increase the likelihood of structural anomalies and clonal variability, underscoring the importance of carefully adjusting cytokinin concentrations to ensure efficiency and fidelity in micropropagation protocols (García-Ramírez *et al.*, 2010; Leão *et al*., 2020). Kim *et al.*, (2023), in a study on grapevines, reported a pattern similar to this study: low concentrations of 6-BAP (≈2 μM) significantly enhanced shoot induction, whereas higher doses inhibited growth and promoted callus formation. Mudoi and Borthakur (2009) documented in Bambusa balcooa that 1.0 mg L^{-1} of 6-BAP produced 8–10 shoots per node, with good proliferation and rooting ability, confirming that cytokinin response depends on optimal concentration range and species-specific factors.

In *G. angustifolia*, Jiménez *et al.*, (2006) reported that 3.0 mg L^{-1} of 6-BAP was sufficient to achieve optimal shoot induction under experimental conditions, a value close to that identified in this study (4.83 mg L^{-1}). The slight variation between both results may be attributed to differences in explant type, physiological age of donor material, and physicochemical properties of the culture medium (semi-solid vs. liquid or temporary immersion systems). In fact, Gutiérrez *et al.*, (2016) demonstrated that temporary immersion systems (RITA®) supplemented with 3 mg L^{-1} BAP produced 2.7 shoots per explant in bamboo, suggesting that the dynamic availability of nutrients and growth regulators in liquid media enhances cytokinin efficiency compared to semi-solid systems. Other studies have also shown that higher BAP concentrations (≥6 mg L^{-1}) can further increase shoot multiplication in liquid media (García-Ramírez *et al.*, 2010; Leão *et al.*, 2022), though such levels may induce somaclonal variation or abnormal morphologies. In contrast, the present research showed that beyond the optimal range, the response declined, aligning with trends reported for tropical bamboos and other woody species.

Furthermore, it is important to highlight that the exclusive use of 6-BAP in this study allowed the isolated assessment of its effect on shoot proliferation. However, recent investigations suggest that combining cytokinins with auxins can substantially enhance morphogenic responses. Mishra *et al.*, (2022) and Sharothi *et al.*, (2022) demonstrated that incorporating naphthaleneacetic acid (NAA) or other cytokinins such as thidiazuron (TDZ) in combination with 6-BAP significantly increased shoot number and improved the morphological quality of regenerated plantlets. Thus, while the present study confirmed 6-BAP's effectiveness as a primary growth regulator, future experiments should explore hormonal interactions to further enhance in vitro multiplication efficiency in *G. angustifolia*.

In comparative terms, differences in maximum shoot numbers among studies highlight the influence of factors such as explant type, basal medium composition, carbon source, physiological condition of donor material, and the culture system employed. While some studies have reported between 8 and 12 shoots per explant after several subcultures (Marulanda *et al.*, 2017; Nogueira, Gomes, and Scherwinski-Pereira, 2019), the maximum mean obtained here was 3.57 shoots in a single culture cycle. This contrast indicates that protocol efficiency could be further enhanced by optimizing subsequent multiplication phases and integrating dynamic systems such as temporary immersion.

Ultimately, the results of this study demonstrate that 6-BAP concentration exerts a significant and nonlinear effect on shoot proliferation in *G. angustifolia*, reaffirming its role as a key regulator in the micropropagation of tropical bamboos. Moreover, the success of the disinfection protocol employed represents a critical element for ensuring explant asepsis and viability—an indispensable requirement for in vitro culture efficiency. Collectively, these findings strengthen the scientific foundation for developing more efficient micropropagation protocols for *G. angustifolia*, contributing to its sustainable utilization and

potential application in reforestation, ecological restoration, and productive development programs in tropical regions.

In this study, the decision to optimize only 6-BAP was based on its well-documented effectiveness as the primary cytokinin for shoot induction in bamboos, allowing a clear and isolated evaluation of its role in *G. angustifolia*. This methodological focus ensured robust statistical modeling and reliable interpretation of results. Nevertheless, recent studies have demonstrated that combining cytokinins with auxins, such as NAA, or with other cytokinins like TDZ, can significantly enhance morphogenic responses and improve shoot quality (Mishra *et al.*, 2022; Sharothi *et al.*, 2022; Shirin *et al.*, 2026). Future research should therefore explore these hormonal interactions to further optimize multiplication efficiency and stability in tropical bamboo micropropagation protocols.

CONCLUSIONS

This study optimized the 6-BAP concentration for the in vitro micropropagation of *G. angustifolia* through a quadratic model derived from RSM. The optimal concentration of 4.83 mg L^{-1} 6-BAP maximized morphogenic response, producing approximately 3.57 shoots per explant. The model exhibited a robust and statistically significant fit (R^2=0.9735; $p<0.0001$), confirming a nonlinear relationship between cytokinin dosage and shoot proliferation. These results provide valuable insights for developing an efficient and reliable large-scale in vitro propagation protocol, contributing to the mass and controlled propagation of this bamboo species.

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