



Optimization of *in vitro* Micropropagation Protocol for *Bambusa vulgaris* Schrad.: Overcoming Contamination and Enhancing Shoot Regeneration

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ABSTRACT

Bambusa vulgaris Schrad., known for its rich cellulose content, serves as an important resource for biofuel production and is widely utilized across diverse industries. It promotes the circular economy and provides a sustainable substitute for fossil fuels. The species has a very high demand in the present scenario and needs to be propagated at a very large scale. The conventional and vegetative propagation methods of the species are inadequate, labour-intensive and slow, resulting in a limited supply of plant material that can't meet the rising demand. This study aims to develop and optimize a standard *in vitro* micropropagation protocol of *Bambusa vulgaris* Schrad. to meet the rising global demand for the species in a short period. Nodal segments of *Bambusa vulgaris* were aseptically cultured on agar solidified MS medium supplemented with various combinations of auxins (NAA, IAA) and cytokinins (BAP) at various concentrations at 25±2°C. Effective antifungal agents were selected through disc diffusion assay and used to pre-treat explants. Data were analyzed using Microsoft Excel, with results expressed as mean ± SD. MS medium supplemented with 2.0 mg L⁻¹ BAP + 0.2 mg L⁻¹ IAA showed the best results, with 100% response and an average of 4.18±1.67 shoots per explant within 20 days. This protocol significantly reduces contamination and promotes rapid, large-scale production of the bamboo species, addressing the limitations of conventional propagation methods.

Key words: Micropropagation, MS medium, Nodal segment, Plant growth regulators, Sterilization.

Bamboo, often called “green gold” or “poor man’s timber,” is a crucial natural and renewable bioresource worldwide. It belongs to the members of the Poaceae subfamily Bambusoideae, with tropical Asia having the most diverse bamboo population with up to 60 genera and 1000 species (Bystrakova, 2003). Bamboo has been a vital component of both human culture and the global economy since time immemorial. The plant has over 4000 traditional uses and 1500 commercial applications (Hsiung, 1988), it provides raw materials used in India's paper and pulp industries, including wood for handicrafts, furniture, scaffolding and equipment for agriculture and pisciculture, as well as fuel for combustion and other bioenergy uses and in food consumption. Bamboo is also beneficial for flood management, soil restoration and carbon sequestration (Laishram *et al.*, 2022; Ogunwusi and Jolaoso, 2012).

The rhizome, culm, bark shavings, shoots, leaves, roots and seeds of the bamboo plant are among the components that are utilized medicinally (Singh *et al.*, 2021; Sreejith *et al.*, 2025). Because of its nutritional value and medicinal potential, bamboo is becoming more and more popular worldwide in the culinary, pharmaceutical and cosmetics sectors (Chakma, 2016). Traditional Asian remedies use its leaves and shoots because of their therapeutic qualities. For thousands of years, traditional medical systems, such as the Ayurvedic system (Chongtham *et al.*, 2011), have employed bamboo. It contains anti-aging

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and anti-stress qualities and is used to treat a variety of illnesses. Bamboo is utilized in several parts of the world as a natural medicine for ailments, including diabetes and malaria, as well as animal feed. Using a leaf extract from *Bambusa vulgaris*, silver nanoparticles with a high degree of crystallinity and a spherical form of 22 nm were effectively produced. These nanoparticles have the potential to treat wastewater because of their surface plasmon resonance, efficient methylene blue dye degradation, enhanced photostability and reusability. Additionally, they demonstrated antibacterial and antibiofilm qualities against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Prabula *et al.*, 2024). Bamboo is a useful feedstock for chemicals and fuel ethanol because of its high sugar

content, which has been demonstrated to have biofuel potential (Yamashita *et al.*, 2010). Because of its rapid growth and abundant lignocellulosic biomass production, it is a good option for biofuel. *Bambusa vulgaris* is a possible source of raw materials for the manufacturing of biofuel, producing bio-oil via pyrolysis that resembles conventional biodiesel in many aspects (Mujtaba *et al.*, 2023).

There is a high demand for *Bambusa vulgaris*, but conventional multiplication methods are slow and inefficient, failing to meet this demand. Although vegetative propagation is more reliable, it is labour-intensive and yields limited results. In-vitro micropropagation offers a viable alternative for the large-scale, rapid production of genetically uniform, disease-free plants. However, existing protocols are not well-optimized, leading to low rates of shoot initiation and proliferation (Malini and Anandakumar, 2013; Gonçalves *et al.*, 2023). Therefore, this study aims to develop and optimize a standard, sterile in-vitro micropropagation protocol to address these challenges and meet global demand efficiently.

The nodal explants of *B. vulgaris* were collected during the month of February, 2024, from the campus of Dibrugarh University, Dibrugarh, Assam, to initiate aseptic cultures in the Plant tissue culture laboratory of the Centre for Biotechnology and Bioinformatics, Dibrugarh University. A thorough surface sterilization protocol was implemented for the bamboo explants to minimize contamination under aseptic conditions in a LAF (Laminar air flow) cabinet. Explants were processed by thoroughly washing under running tap water for 15 minutes, followed by a 20-minute wash with 0.1% Tween-20 solution. The explants were then

cut into 4 cm (node) segments, soaked in 70% ethanol for 5 minutes, treated with 0.1% sodium hypochlorite for 15 minutes and finally with 1% mercuric chloride for 10 minutes. After each step, the explants were rinsed thoroughly with sterile water to remove any residual chemicals. After sterilization, the explants were inoculated into solid MS (Murashige and Skoog) medium prepared by adding 8 gL⁻¹ agar supplemented with different concentrations and combinations of plant growth regulators, viz., Indole-3-acetic acid (IAA), Naphthalene acetic acid (NAA) and 6-Benzylaminopurine (BAP) (Table 1). The pH of the media was adjusted to 5.8±0.1. The explants were carefully positioned in the medium and transferred to an incubation room maintained at 25±2°C with a 16-hour photoperiod at 2500 lux. Cultures were monitored daily to record shoot initiation, growth and length. After observing the growth of shoots, the percentage of culture establishment was calculated using the formula:

$$\text{Establishment (\%)} = \frac{\text{No. of plants established}}{\text{Total no. of explant}} \times 100$$

However, certain microbial contamination was observed in cultured samples. Contaminants from bamboo explant cultures were inoculated on Sabouraud Dextrose Agar (SDA) medium to isolate and identify the contaminants. The inoculated samples were incubated at 25±0.5°C for 96 hours to observe microbial growth. Two distinct colonies were then isolated from the culture based on their morphology, cell shape, arrangement and staining characteristics, with similar colonies grouped as a single type of colony-forming unit (CFU) (Fig 1). The identification of fungal contaminants was performed using lactophenol cotton blue (LPCB) staining, which allowed clear observation of fungal structures. The staining procedure was done according to the method of Leck (1999). The morphological characteristics of microbial colonies isolated from samples showed a single colony characterized by white in color, flat in elevation, with filiform margins, filamentous form and a cottony surface texture. These features suggest that the colonies from both samples are morphologically similar, possibly indicating

Table 1: Combinations of plant growth regulators.

Treatment no.	Hormonal combination (mg L ⁻¹)		
	BAP	NAA	IAA
Com1	2.0	0.2	-
Com2	2.0	-	0.2
Com3	2.0	0.2	0.2

Com1-Com3 are the growth regulator combinations.

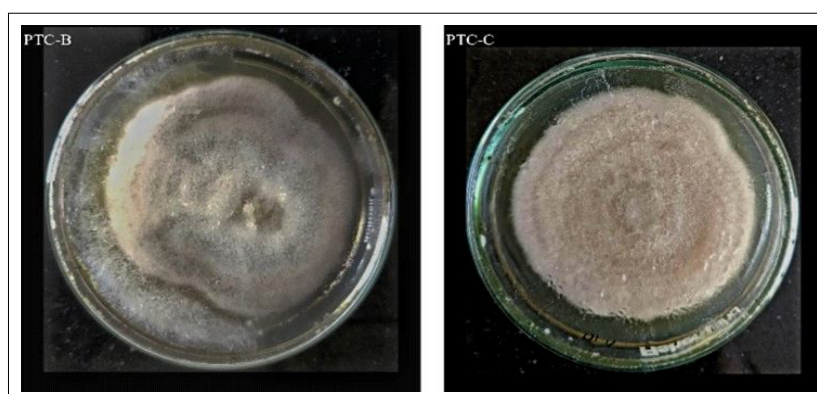


Fig 1: Colonies of isolates on SDA media.

the presence of the same or closely related fungal species. After staining and morphological characterization, the isolates were found to be *Aspergillus* species, although confirmation of the species has yet to be performed through gene sequencing.

To address the contamination, antimicrobial susceptibility tests were conducted using the disc diffusion method following Bauer and Kirby, 1966. Sterile discs soaked in different concentrations of various antifungal agents were placed on SDA plates pre-inoculated with the desired fungal isolate and incubated for 36 hours. After incubation, zones of inhibition around each disc were measured to determine the effectiveness of the antifungal treatments against the isolated fungal strains (Table 2). Based on the results, the effective antifungal agents were selected for further use in culture. After identifying the proper antibiotics, bamboo explants were treated overnight with an antimicrobial solution containing Ridomil (500 mg L⁻¹), Indofil (500 mg L⁻¹), Syscon (300 mg L⁻¹) and Streptocycline (0.25 mg L⁻¹).

All experimental data were analyzed using Microsoft Excel. The results are presented as mean $\pm$ standard deviation (SD), calculated from at least three biological

replicates per treatment. Pearson's correlation coefficient (*r*) was calculated to assess the relationship between photoperiod duration and shoot length.

The shoot initiation was observed 14 days after inoculation. It was observed that the combination of BAP and IAA (Table 3) yielded the most successful shoot induction, with a 100% bud break and shoot formation rate across all tested explants (Table 3; Fig 2B and Fig 3). This combination produced an average of 4.18 $\pm$ 1.67 shoots per explant, significantly outperforming the other combinations (Table 3). The response rate was significantly lower at 33.34% when BAP and NAA was combined (Fig 2A), but it increased to 66.67% when IAA was added (Table 3, Com3), with an average of 3.17 $\pm$ 2.84 shoots per explant (Table 3 and Fig 2C) demonstrating the importance of IAA in improving shoot initiation in combination with BAP. Our findings corroborate with *B. arundinacea* (Retz.) Wild in which a combination of BAP (3.0 mg L⁻¹) and IBA (0.5 mg L⁻¹) showed the highest shoot bud initiation (Venkatachalam *et al.*, 2015). Similarly, a combination of BAP, IAA, NAA, along with 2,4 D in MS medium was found to be most effective in inducing bud break and multiple shoot formation in *B. vulgaris* (Kaladhar *et al.*, (2017). Although both methods were effective, we were able to achieve a greater shoot induction rate with 2.0 mg L⁻¹ BAP + 0.2 mg L⁻¹ IAA. In line with our findings Malini and Anandakumar, 2013 achieved shoot multiplication of *Bambusa vulgaris* by combining BAP and kinetin.

Similarly, Kalaiaresi *et al.* (2014) achieved approximately 91.5% shoot bud induction in *Bambusa arundinacea* using 3.0 mg/L BAP and 0.5 mg/L kinetin. Another study obtained successful shoot initiation with 3.0 mg/L BAP and 0.5 mg/L IBA, reaching about 87.2% induction and an average of 24 shoots per explant (Venkatachalam *et al.*, 2015). In our study, the protocol using 2.0 mg/L BAP and 0.2 mg/L IAA in *Bambusa vulgaris* resulted in a high frequency of shoot induction. These findings demonstrate that effective shoot bud formation can be achieved across various *Bambusa*

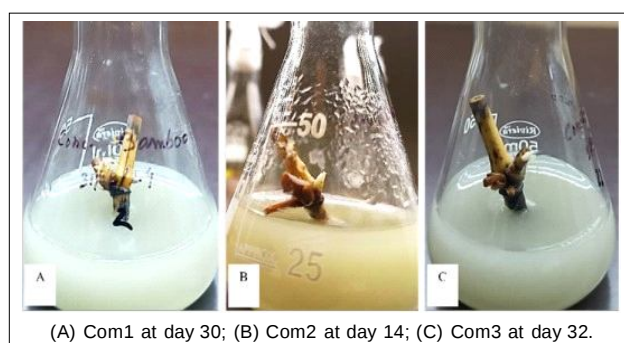


Fig 2: Shoot buds initiation from explants with different growth regulator combinations in MS media.

Table 2: Antifungals showing average zone of inhibition against the isolates.

Isolates	Antibiotics					
	Bavistin	Ridomil	Indofil	Saaf	Syscon	Mixture (BRISS)
Isolate B	-	+	+	-	-	+
Isolate C	-	+	+	+	+	+

'+' indicates zone of inhibition and '-' indicates no zone of inhibition.

Table 3: Effect of different plant growth regulators used in media on shoot initiation.

Treatment no.	Hormonal combination (mg/L)			No. of days for initiation (in days)	Percentage of response	No. of shoot per explant
	BAP	NAA	IAA			
Sample 1	2.0	0.2	-	30	33.34%	0.33 $\pm$ 0.57
Sample 2	2.0	-	0.2	14-28	100%	4.18 $\pm$ 1.67
Sample 3	2.0	0.2	0.2	25-30	66.67%	3.17 $\pm$ 2.84

The values in the table were after 28 days of observation (n = 5).

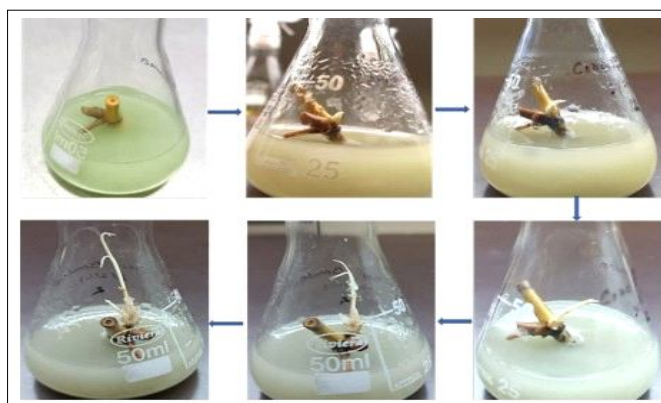


Fig 3: Shoot growth in explant 1 in MS media with BAP 2.0 mg/L + IAA 0.2 mg/L from day 0 to day 42.

species using different combinations and concentrations of plant growth regulators (Harb *et al.*, 2010). Such differences emphasize the importance of tailoring PGR concentrations to individual species for efficient and cost-effective micropropagation.

The study further proceeded with the combination of BAP and IAA and shoot growth was monitored over 42 days, revealing a consistent increase in shoot length. The average shoot length increased from 1.5 ± 0.5 cm at 21 days to 4.57 ± 1.88 cm by 42 days (Fig 2 and 3). The consistent growth trend was further influenced by the photoperiod. Under a 16-hour photoperiod, shoots averaged 1.43 ± 0.73 cm in length, while increasing the light exposure to 18 hours per day resulted in longer shoots averaging 2.57 ± 1.30 cm. The most significant growth was observed under continuous light (24 hours per day), where shoots reached an average length of 4.57 ± 1.88 cm. The strong positive correlation between photoperiod and shoot length, confirmed by a Pearson correlation coefficient of 0.99, suggests that extended light exposure significantly enhances shoot growth.

Root induction was performed by transferring regenerated shoots from the shoot induction medium to a root induction medium composed of half-strength MS medium with various concentrations of IBA and BAP: $\frac{1}{2}$ MS + 2 mg/L IBA, $\frac{1}{2}$ MS + 2 mg/L IBA + 0.5 mg/L BAP, $\frac{1}{2}$ MS + 2.5 mg/L IBA (Indole butyric acid) + 0.5 mg/L BAP and $\frac{1}{2}$ MS + 3 mg/L IAA + 1 mg/L BAP under incubation on 16/8 hour photoperiod. This step was crucial for the successful establishment of complete plants. The size of explants and careful optimization of sterilization techniques and photoperiod were crucial for successful culture initiation and growth.

CONCLUSION

The study has contributed to the micropropagation of *Bambusa vulgaris* for large-scale commercial production by establishing a standard procedure for sterilization, along with an effective antibiotic combination, leading to a lower contamination rate. Additionally, optimizing the Plant growth

regulators and refining culture conditions resulted in efficient shoot regeneration. The present study found that shoot proliferation was best in MS medium supplemented with 2.0 mg L^{-1} BAP + 0.2 mg L^{-1} IAA. However, further studies are essential to validate our study's findings and increase the reproducibility of our experiments. The protocol holds potential for crop improvement by obtaining disease-free plantlets, important secondary metabolites production and standardization for maximizing the micropropagation of other *Bambusa* sp. varieties, with implications for the pharmaceutical and biorefinery industry.

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Author contributions

The conceptualization of the research was done by Rasmita Khatonier. The design of the study was carried out by Rasmita Khatonier and Jyotish Sonowal. Laboratory work was primarily conducted by Jahnabi Dutta, with additional support from Pranit Saikia, Jyotish Sonowal. The data interpretation was performed by Bhaskar Jyoti Saikia, Abhisek Dasgupta and Pranit Saikia. The manuscript was written by Jahnabi Dutta, Rasmita Khatonier and Jyotish Sonowal. All authors have read and approved the final manuscript.

Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or

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