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Prajwal D
M.Sc. Agriculture Scholar, Department of Agricultural Botany, College of Agriculture, Pune, Mahatma Phule Krishi Vidyapeeth, Maharashtra, India

Dr. PY Shinde
Assistant Professor, Department of Agricultural Botany, College of Agriculture, Pune, Mahatma Phule Krishi Vidyapeeth, Maharashtra, India

Dr. MR Bhakre
Junior Research Fellow, Department of Agricultural Botany, College of Agriculture, Pune, Mahatma Phule Krishi Vidyapeeth, Maharashtra, India

Dr. TD Katore
Junior Research Fellow, Department of Agricultural Botany, College of Agriculture, Pune, Mahatma Phule Krishi Vidyapeeth, Maharashtra, India

Dr. RD Nimbalkar
Associate Professor of Agricultural Botany, Agricultural Research Station, Karad, Satara, Maharashtra, India

Corresponding Author:
Prajwal D
M.Sc. Agriculture Scholar, Department of Agricultural Botany, College of Agriculture, Pune, Mahatma Phule Krishi Vidyapeeth, Maharashtra, India

Development of shoot initiation protocol for *in vitro* regeneration in bamboo through nodal segments (*Bambusa balcooa* Roxb.)

Prajwal D, PY Shinde, MR Bhakre, TD Katore and RD Nimbalkar

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Abstract
Bamboo propagation is typically carried out using two primary methods: one involves the use of offsets, culm, or side branch cuttings, while the other relies on micropropagation, also known as clonal propagation, which is most effectively achieved through tissue culture techniques. *In vitro* propagation is particularly useful to meet the growing demand for bamboo species, given their diverse applications. This study focused on developing a sterilization and initiation protocol for *in vitro* regeneration of *Bambusa balcooa* Roxb. The experiment was conducted using nodal explants collected from the Research Farm at the College of Agriculture, Pune, and the tissue culture work was carried out in the Biotechnology Laboratory at the same institution. Nodal explants, when cultured on MS (Murashige and Skoog) media, proved to be ideal for *in vitro* regeneration. The sterilization protocol for the nodal explants, which included treating them with Tween 20 (10 min), 1% Bavistin (7 min), 0.1% $HgCl_2$ (5 min), 5% sodium hypochlorite (5 min), and 70% ethanol (1 min), followed by three rinses in distilled water between each treatment, resulted in the lowest contamination rate of 13.3%. Various plant growth hormones were tested in MS media to assess their effects on shoot initiation. Among the treatments, BAP (3 mg/l) yielded the best results, with 83.3% of the explants showing shoot initiation as early as 8 days after inoculation.

Keywords: Nodal explants, sterilization, initiation, MS media, standard error

Introduction
Bamboo, a member of the Poaceae family, is known for its rapid growth and impressive size. Some bamboo species can reach heights of up to 36 meters and have diameters ranging from 1 to 35 cm. As one of the fastest-growing plants, certain species can grow 30 to 100 cm per day. Bamboo is ecologically significant because its roots absorb more water than many other plants, helping to prevent soil erosion. Additionally, it absorbs carbon dioxide (CO_2) and releases oxygen into the atmosphere. Economically, bamboo is valuable in both rural and urban settings, serving as a source of building materials, animal feed, and paper. China is the world's largest bamboo producer. Bamboo species, which belong to approximately 75 genera and include around 1,250 species, are distributed globally, with the majority found in tropical regions, though they also grow in subtropical and temperate climates (Mudoi *et al*., 2018) [6].

Bambusa balcooa, a versatile bamboo native to the Indian subcontinent, typically grows between 12 and 23 meters tall, with a diameter of 18 to 25 cm, thriving at altitudes up to 600 meters. Its flowering cycle is unique, occurring once every 55 to 60 years, after which the plant dies without producing seeds (Tewari, 1992) [12]. Bamboo's natural reproduction is often hindered by various challenges, such as short seed dormancy, high seed sterility, low seed viability, susceptibility to seed-borne diseases, and predation by wildlife, particularly rodents (Rajput *et al*., 2019) [9]. Known as "tree grasses," woody bamboos play crucial ecological and cultural roles in many parts of Asia, Africa, and the Americas. Due to their rapid growth, strong rhizomes, and durable culms, bamboos provide a sustainable alternative to timber, offering a wide array of economic, environmental, and social benefits. Bamboo is also seen as a key plant for conservation efforts, helping to mitigate the impacts of climate change (Liese and Kohl, 2015) [5].

While *Bambusa balcooa* can be propagated vegetatively using culms, rhizomes, or branches, these methods are often inefficient for large-scale propagation due to the bulky nature of the propagules, seasonal variations, and poor rooting success (Pattanaik *et al*., 2004) [8]. Recent advancements in micropropagation using axillary shoots have shown promising results for the *in vitro* propagation of *B. balcooa*. Studies by Das and Pal (2005) [3], Islam and Rahman (2005) [4], and Ansari *et al*. (2017) [1] have highlighted the potential of this technique. *In vitro* propagation offers numerous advantages, such as year-round production of healthy, disease-free plants, the ability to preserve germplasm over long periods, and the facilitation of international exchange of pathogen-free plant material (Sun *et al*., 2008) [11].

This study presents a micropropagation protocol for *Bambusa balcooa* using nodal segments, which offers a promising approach for large-scale propagation. This method could aid in land reclamation efforts through bamboo planting and provide valuable support for rural economies and livelihoods.

Materials and Methods

All chemicals used in the current study were of analytical grade. Murashige and Skoog (MS) medium was employed for the *in vitro* regeneration of bamboo throughout the research. Stock solutions of the individual components were prepared first, and then combined to formulate the medium, ensuring the final concentrations were properly balanced. Stock solutions of plant growth hormones were prepared by dissolving the required amounts of auxins (IAA and NAA) in a few drops of absolute alcohol, while cytokinins (BAP and Kn) were dissolved in 10 mL of 1N NaOH. The final volumes were adjusted by adding double-distilled water to bring the total to 1 liter. The stock solutions of growth regulators were stored in a refrigerator and used within two weeks of preparation. Double-distilled water was used for all experimental procedures. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl before autoclaving. Plant growth hormones were added to the basal medium before autoclaving. The MS medium served as the basal medium for all stages of the experiment. Borosilicate glassware was used throughout the study. The glassware was thoroughly cleaned with Tween 20 biodegradable detergent, followed by washing with tap water to remove any detergent residues. Finally, the glassware was rinsed with distilled water and sterilized in an oven at 160–180 °C for 3-4 hours.

Autoclaving: Sterilization of media was done in autoclave. Micronutrient combinations, distilled water, and other stable mixtures prepared were autoclaved. Autoclaving the culture media contained in glass containers were carried out at 15 psi. pressure and 121 °C temperature for about 15- 40 minutes. Exposure time for autoclaving is dependent on volume of liquid which is needed to be sterilized. Maximum care was taken during the procedure. After the media is autoclaved, it was stored in dark environment for 48 hours at 25±2 °C.

Culture room sterilization and transfer area: Initially, cleaning of culture rooms was done by gently washing the floors and walls by using a detergent soap and then cleansing with phenyls daily. Sterilization of transfer area was done by exposing it to UV light. Installation of HEPA filter ventilation unit checked that aseptic condition of transfer area is maintained. Sterilization of laminar airflow hoods were done by wiping the working surface using 70% ethyl alcohol.

Sterilization of ex-plant: Obtaining sterilized explants is complicated because during the sterilization process biological activity of living material should be maintained and only the fungal and bacterial contaminant needs to be eliminated. Various surface sterilization agents were used for sterilization of explants. Nodal segment with 2-3 nodes were taken and used as explants for studying the effect of sterilization. Further trimming of the collected explants to desired sizes with sterile scalpel blade was done. Once the explants were cut to suitable size having a single node, they were washed well in the running tap water for about 20 minutes and then they were treated with or without different sterilants (Washing 3 times with sterile distilled water between each step) for studying their effects on sterilization and treatment numbers were given from S1 to S6.

Inoculation of explants: Inoculation of the explants on culture media aseptically was done after sterilization process. For doing inoculation, the explants were shifted to large sterilized glass petri plates by using sterile forceps done under strict aseptic conditions. These explants were then inoculated vertically to culture bottles which contain MS medium added with different plant growth hormones. Once the explants are vertically inoculated in culture by keeping the node above medium, the culture bottle's mouth was quickly flamed and closed with cap.

Culture conditions: All the cultures were incubated at a temperature of 25±2 °C under fluorescent light in 16: 8 hour's photoperiod and light intensity of 2500 lux.

Effect of plant growth hormones on shoot initiation: Three types of hormone combinations were incorporated in media. Each type is done in different hormone concentrations as follows:

(a) BAP added singly in 1 L medium

BAP (0.5 mg/l, 1 mg/l, 2 mg/l, 3 mg/l, 4 mg/l, 5 mg/l)

(b) Kinetin added in 1 L medium

Kinetin (0.5 mg/l, 1.0 mg/l, 2.0 mg/l, 3.0 mg/l, 4.0 mg/l, 5.0 mg/l)

After around 3 weeks from shifting explants to inoculation media, number of days required for shoot initiation and sum total of shoots formed for explants were recorded.

Observations recorded: Each treatment was replicated 3 times and then the whole experiment was repeated twice again for getting unbiased results. Observation of cultures were done periodically and following recording of observations were done: Observations recorded: Each treatment was replicated 3 times and then the whole experiment was repeated twice again for getting unbiased results. Observation of cultures were done periodically and following recording of observations were done: Observations recorded: Each treatment was replicated 3 times and then the whole experiment was repeated twice again for getting unbiased results. Observation of cultures were done periodically and following recording of observations were done:

1. Effect of treatment of nodal explants with various sterilants.
2. Shoot initiation percentage.
3. Number of shoots/explant.
4. Number of days required for shoot initiation

Completely randomized design was used for conducting the experiment. Data for initiation of explants collected were

analysed for mean and standard error as given by Snedecor and Cochran (1972) [10].

Results and Discussion

The experiment entitled "*In-vitro* regeneration in Bamboo through nodal segments (*Bambusa balcooa* Roxb.) was carried out in a Biotechnology Laboratory, College of Agriculture, Pune-5 during the year 2023-2024. The development of an efficient *in vitro* regeneration protocol for Bamboo using nodal segment as explant was carried out. The nodal segment explant was taken from the healthy plants of Bamboo from Botany Division Research Farm, College of Agriculture, Pune-05. The preparation of explant, sterilization and inoculation done according to methodology provided in the chapter of materials and methodology.

Effect of Sterilization

Sterilization of nodal explant carried out by using six different treatments, treatment number S1 showed the highest contamination percentage of 46.7 and S6 showed the lowest contamination percentage of 13.3 as shown in Table 1

Thus, the following procedure of S6 was used for sterilization of explant during research. After the explants were cut into suitable size having a single node, the explants were sterilized using a multi-step procedure, starting with a 20 minutes wash in running tap water, followed by a 10 minutes wash with Tween-20 detergent and vigorous shaking. Afterwards, the explants were rinsed with tap water for 5 minutes to remove any remaining detergent. Next, the explants were treated with 1% Bavistin for 7 minutes and then rinsed three times with double distilled water. The explants were then washed three times with sterile double distilled water to ensure complete removal of any contaminants. Surface sterilization was achieved by treating the explants with 0.1% $HgCl_2$ for 5 minutes, followed by a 5 minutes treatment with 5% NaOCl. The explants were then rinsed 3-4 times with sterilized double distilled water to remove any residual sterilizing agents. Finally, the explants were sterilized with 70% ethanol for 1 minute and rinsed three times with double distilled water before being inoculated onto culture media containing various plant growth regulators. Surface sterilized explants and inoculation of explants are shown in Plate1

Table 1: Effect of treatment of nodal explants with various sterilants.

Treatment Number	Treatment details	% of explant showed initiation without any contamination (a)	Contamination percentage (b), where, b= 100-a
S1	Tween 20 (5 min) + 1% Bavistin (5 min) + 0.1% $HgCl_2$ (2 min)	53.3	46.7
S2	Tween 20 (7 min) + 1% Bavistin (7 min) + 0.1% $HgCl_2$ (2 min)	60.0	40.0
S3	Tween 20 (10 min) + 1% Bavistin (10 min) + 0.1% $HgCl_2$ (2 min)	66.7	33.4
S4	Tween 20 (5 min) + 1% Bavistin (5 min) + 0.1% $HgCl_2$ (5 min) +5% NaOCl (5 min)	73.3	26.6
S5	Tween 20 (7 min) + 1% Bavistin (5 min) + 0.1% $HgCl_2$ (5 min) +5% NaOCl (5 min) + 70% ethanol (30 sec)	80.0	20.0
S6	Tween 20 (10 min) + 1% Bavistin (7 min) + 0.1% $HgCl_2$ (5 min) +5% NaOCl (5 min) + 70% ethanol (1 min)	86.7	13.3

Plate 1: Inoculation of sterilized explants on semi solid MS medium

Effect of BAP for Shoot Initiation

The BAP hormone was used at different concentrations for initiation of nodal explant. The nodal segments were inoculated on MS media with different hormone concentrations and observations were recorded (Table 2). Treatment names were given as IM 1-6 (Initiation media 1-6). For shoot initiation from nodal segment took place within 8 to 12 days. The BAP 3.0 mg/l (IM 3) showed higher shoot initiation 83.3% and 8.00±0.25 days required for shoot initiation. The same treatment (BAP 3.0 mg/l) recorded maximum number of shoots per explant 3.53±0.21. Hence, BAP 3.0 mg/l (IM 3) was considered as best treatment for shoot initiation shown in Plate 2. The present results are also in agreement with the results obtained by Chavan *et al*., (2021) [2] optimized the *in vitro* propagation protocol from nodal culture of *Bambusa balcooa*. The investigation discovered that highest number of shoots per explant were obtained on MS medium supplemented with 4.0 mg/l BAP.

Table 2: Effect of BAP on shoot initiation of *Bambusa balcooa* nodal explants on MS medium

Treatment Name	PGR Concentration of BAP (mg/l)	% of nodes initiated shoot	No. of days required for shoot Establishment (Mean ± SE)	No. of shoots per Explant (Mean ± SE)
IM 1	1.0	58.3	12.00±0.51	1.50±0.14
IM 2	2.0	66.6	11.00±0.44	2.43±0.12
IM 3	**3.0**	**83.3**	**8.00±0.25**	**3.53±0.21**
IM 4	4.0	75.0	9.00±0.25	2.90±0.18
IM 5	5.0	66.6	10.66±0.29	2.76±0.29
IM 6	6.0	41.6	11.66±0.64	1.93±0.10

Plate 2: Shoot initiation of nodal explants on semi solid medium containing BAP (3 mg/l)

Effect of Kinetin for Shoot Initiation

The kinetin hormone was used at different concentrations for initiation of nodal explant. The nodal segments were inoculated on MS media with different hormone concentrations and observations were recorded (Table 3). Treatment names were given as IM 7-12 (Initiation media 7-12). For shoot initiation from nodal segment took place within 10 to 13 days. The Kinetin 4.0 mg/l (IM 11) showed higher shoot initiation 75% and 10.00±0.19 days required for shoot initiation. The same treatment (Kinetin 4.0 mg/l) recorded maximum number of shoots per explant 2.46±0.10. Hence, Kinetin 4.0 mg/l (IM 11) was considered as best treatment for shoot initiation shown in Plate 3. The results obtained for number of shoots are in par with the results recorded by Rajput *et al*., (2019) [9]. They also studied on MS medium and recorded that Kinetin concentrations significantly affected by the number of shoots.

Table 3: Effect of Kinetin on shoot initiation of *Bambusa balcooa nodal* explants on MS medium.

Treatment Name	PGR Concentration of Kinetin (mg/l)	% of nodes initiated shoot	No. of days required for shoot Establishment (Mean ± SE)	No. of shoots per Explant (Mean ± SE)
IM 7	0.5	33.3	13.00±0.12	1.40±0.13
IM 8	1.0	41.6	12.00±0.23	1.56±0.11
IM 9	2.0	58.3	11.86±0.16	1.86±0.12
IM 10	3.0	66.6	10.90±0.22	2.06±0.11
IM 11	4.0	75.00	10.00±0.19	2.46±0.10
IM 12	5.0	66.6	12.60±0.13	2.26±0.18

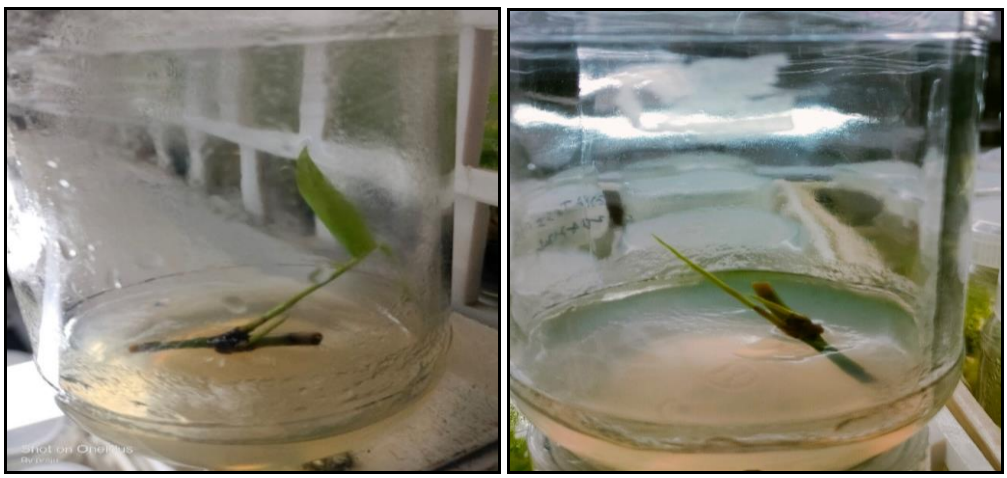

Plate 3: Shoot initiation of nodal explants on semi solid medium containing Kinetin (4 mg/l)

Summary and Conclusion

Bambusa balcooa is recognized as a commercially significant edible bamboo species by the National Mission on Bamboo Application (NMBA). This species is important not only as a food source but also as a raw material for household construction and the production of various agricultural tools. It is primarily cultivated in homestead gardens, and promoting its commercial cultivation can drive economic growth for rural communities in the northeastern states.

Some of the various bamboos, *Bambusa balcooa* is one of the most important and desired species by farmers for plantation cultivation, owing to its multipurpose utility. The species has been extensively cultivated for an unknown period of time and has become a popular bamboo among farmers. It thrives in tropical humid conditions. It also has a high calorific value and Fuel Value Indices (FVI), making it ideal for bioenergy plantations and gasification. *Bambusa balcooa* is ideal for the paper and pulp industry. It has superior strength properties, including adequate culm wall thickness. The life cycle of a clump is more than 50 years, and it has the added benefit of having minimal pest problems and no gregarious flowering in India.

Due to the growing demand for *Bambusa balcooa*, *in vitro* regeneration of this bamboo has become increasingly important because it allows for mass multiplication in a short period of time, thereby despite the limitations of traditional propagation methods previously used.

The current study discovered an effective protocol for *in vitro* development of *Bambusa balcooa. In vitro* cultures of *Bambusa balcooa* were developed by using a nodal segment as an explant and varying combinations of plant growth regulators with MS basal medium for shoot initiation.

For nodal explant sterilization, the procedure involving Tween 20 (10 min), 1% Bavistin (7 min), 0.1% $HgCl_2$ (5 min), NaOCl (5 min), and 70% ethanol, with three washes with distilled water between each sterilant, yielded the lowest contamination rate of 13.3% compared to other treatments. This method was subsequently adopted for all explant sterilizations in the research.

For shoot initiation, MS medium supplemented with 3.0 mg/l BAP yielded the best results, with 83.3% of explants initiating shoots as early as 8 days after inoculation.

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