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Sterilization protocol for *in-vitro* propagation of *Bambusa wamin* and *Dendrocalamus asper*

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Abstract

Bamboo represents a crucial natural resource in tropical and sub-tropical regions, serving numerous commercial purposes. However, escalating demands coupled with challenges such as overexploitation, low productivity in bamboo forest plantations, and insufficient planting stocks have led to a substantial increase in market demand. Micropropagation emerges as a vital solution for the urgent replenishment of diminishing bamboo resources. While bamboo tissue culture holds significant potential for rapid propagation and species conservation, the pervasive issue of endogenous contamination poses a notable obstacle to its successful implementation. Microbial contamination is a serious problem in the cultures of woody plants. The study aimed to develop a sterilization protocol for raising aseptic cultures and facilitate propagation through shoot multiplication. This paper evaluates the sterilization methods to determine the relative effectiveness of various disinfectants. The contamination rate for nodal explants of *Bambusa wamin* and *Dendrocalamus asper* was analyzed with five different treatments (I-V) of fungicide and antibiotic including bavistin, streptocyclin, rifampicin and ciprofloxacin followed by surface disinfection with ethanol and mercuric chloride. The explant treatment V with 500 mg/100 ml bavistin, 100 mg/100 ml streptocycline and 100 mg/100 ml rifampicin, then followed by 500 mg/100 ml streptocycline, 250 mg/100 ml ciprofloxacin and 100 mg/100 ml rifampicin were reported to reduce maximum contamination by 22-36% in both the species. Shoot multiplication serves as a rapid mode of propagation through tissue culture, wherein the sterilization protocol plays a critical role in controlling endogenous or latent contamination, a significant bottleneck in bamboo tissue culture.

Keywords Bamboo; in-vitro; micropropagation; contamination; antibiotic

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1. Introduction

The demand for large-scale propagation of commercially valuable bamboos is ever-growing due to their versatile applications and sustainable nature (Motghare et al. 2023). There is a pressing need to enhance plantation productivity by implementing genetic improvement initiatives and employing advanced management practices in both natural bamboo habitats and cultivated plantations (Singh 2009). Urgent measures are required for the rapid proliferation of these vital resources. Traditional seed propagation techniques are unreliable due to the irregular and unpredictable flowering patterns of bamboo species, which can extend from 7 to 120 years. Furthermore, seeds are susceptible to rapid deterioration in viability, as well as vulnerability to pest infestation and disease during storage in warm and humid environments. In contrast, vegetative propagation methods such as culm, rhizome, or nodal cutting rooting are relatively slow processes. Consequently, plant tissue culture emerges as a pivotal tool for expediting the clonal multiplication of superior plants, enhancing crop genetics, facilitating genetic transformation, and supporting conservation endeavors (Motghare et al. 2023). However, microbial contamination pose significant challenges for in-vitro propagation (Ahmad et al. 2023). Effective sterilization protocols are crucial for ensuring healthy explant survival and subsequent plantlet regeneration, yet optimizing these protocols for each species remains critical.

In vitro cultivation of woody plants is commonly acknowledged as more challenging compared to herbaceous species. This difficulty stems from several factors, including the reduced responsiveness of woody species to Plant Growth Regulators (PGRs) and the ineffectiveness of standard surface sterilization techniques in achieving truly aseptic cultures. Microbial contamination poses a significant issue in woody plant cultures, for which efficient eradication methods are currently lacking. Repeated treatments with aqueous sodium hypochlorite or other disinfectants often result in more harm to the plant material than to the bacteria (Thomas 2004). The nutrient media utilized for plant tissue cultivation serves as an ideal nutrient source for microbial growth, leading to detrimental competition with plant tissues for nutrients. The presence of these microbes in plant cultures typically leads to increased culture mortality, tissue necrosis, diminished shoot proliferation, and rooting (Odutayo et al. 2007), thereby compromising the efficacy of plant cell/tissue culture systems. To minimize infection, tissue culture techniques typically involve growing stock plants under conditions designed to reduce microbial contamination and treating plant materials with various disinfecting chemicals (Kane 2003). Chemical methods employed for eliminating culturable fungal and bacterial

contaminants includes antibiotics, fungicides, alcohols, mercuric chloride, and oxidizing biocides such as halogen compounds, selection of which depends on factors including plant species, type of explants, phytotoxicity considerations, type of contaminants, and cost (Niedz and Bausher 2002).

Bamboo nodal explants, characterized by large intercellular spaces and wide vessel cavities at cut ends, are particularly susceptible to penetration by contaminating agents. These contaminants, deeply embedded within tissues, often evade conventional disinfection treatments, resulting in culture contamination following prolonged inoculation periods (Thakur and Sood 2006). This paper investigates and compares the efficacy of various sterilization protocols for *Bambusa wamin* and *Dendrocalamus asper*. The applications of different combinations of disinfectants, their concentrations, and exposure durations to eliminate microbial contaminants while ensuring optimal explant survival and subsequent plantlet development.

2. Material and Methods

The explants of *Bambusa wamin* Camus, and *Dendrocalamus asper* Scult., used for micropropagation were collected from the Bambusetum of P.N. Mehra, Botanical Garden, Panjab University, Chandigarh, India. The nodal explants were cut from the secondary and tertiary branches of mother plants for both the species. The small single nodal segments (3-4 cm length) each containing an unsprouted bud (Fig.1) were cut, wiped with 70% ethanol and bud prophylla were removed with the help of a blade. It was transfered to a conical flask containing dilute 0.1% solution of surgical hand wash (chlorhexidine gluconate solution) and sterilized for 10 min. The explants were washed with running tap water for 5 min and then treated with 5 different treatment using various fungicide and antibiotic concentration (Table 1).

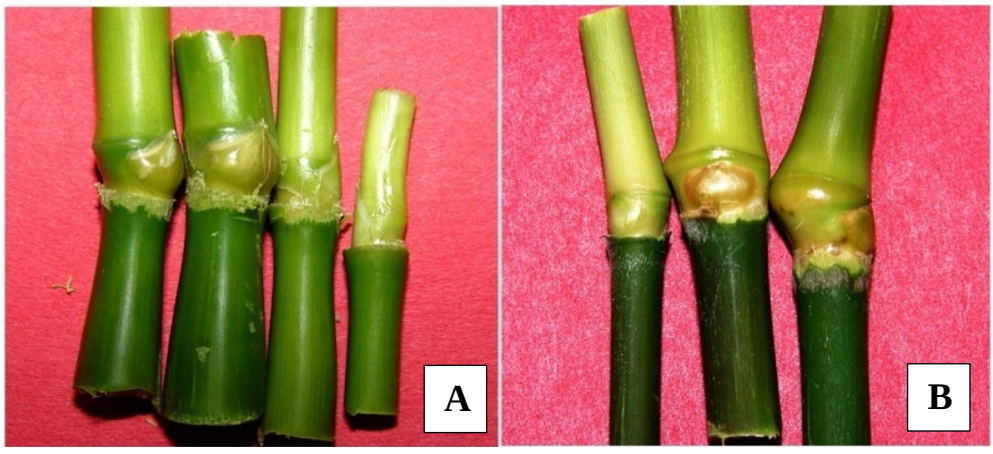

Fig. 1. Nodal explant A) *Bambusa wamin*; B) *Dendrocalamus asper*

Table 1. Explant treatment for aseptic cultures.

Before surface sterilization	Treatment					
Fungicide/Antibiotic (mg/100ml)	I	II	III	IV	V	
					a	b
Bavistin	80	100	120	140	500	-
Streptomycin	40	40	60	100	100	500
Tetracyclin	-	60	-	-	100	-
Rifampicin	-	-	-	-	-	100
Ciprofloxacin	-	-	-	-	-	250
Tween20	2-3drops	2-3drops	2-3drops	2-3drops	2-3drops	
Surface sterilization (mg/100ml)						
Ethanol	70%	70%	100%	100%	70%	
Mercuric Chloride	40	40	100	100	100	

After the treatment, the explants were rinsed twice with autoclaved distilled water which was then surface sterilized with 70% and 100% ethanol for 1 min and rinsed twice with autoclaved distilled water. It was follwed by surface sterilization of $HgCl_2$ for 5-10 min, which was rinsed 5 times to ensure that no traces of mercuric chloride were left. The sterilized explants were inoculated in autoclaved ½ strength MS medium (Murashige and Skoog 1962), with 3% sucrosed, 5.8 pH and 0.8% agar. The data were recorded between two to four weeks post-inoculation for the number of cultures producing shoots and the number of contaminated cultures. For each species, 25 test tubes were inoculated with 3 replictes. Data were calculated as percentages (%) of cultures in terms of contamination, bud break and phytotocicity by using the formula given below.

$$\text{Percent bud break} = \frac{\text{Number of cultures showing bud break}}{\text{Total number of cultures inoculated}} \times 100$$

$$\text{Percent contaminated culture} = \frac{\text{Number of contaminated cultures}}{\text{Total number of cultures inoculated}} \times 100$$

$$\text{Percent dead brown culture} = \frac{\text{Number of dead brown cultures}}{\text{Total number of cultures inoculated}} \times 100$$

3. Results and Discussion

The efficacy of different concentrations of fungicide and various antibiotics in disinfecting nodal explants of *B. wamin and D. asper* was analysed in 5 different treatments (Table 2). In this study, the infiltration of surface contaminants into the extensive spaces of single-node explants was prevented by initially swabbing the explants with 70% ethanol, followed by a 10-

minute wash with a 0.1% solution of surgical hand wash (chlorhexidine gluconate solution). This procedure effectively decreased the likelihood of surface contaminants penetrating the intercellular spaces of nodal explants. Chlorhexidine gluconate solution functions as an antiseptic and antimicrobial skin cleanser with bactericidal properties effective against a broad spectrum of microorganisms, encompassing both gram-positive and gram-negative bacteria such as *Pseudomonas aeruginosa* (Peterson et al. 1978). According to Thakur and Sood (2006), the cut ends of single-nodal explants act as entry points for the deep infiltration of sterilizing agents into the tissue, leading to prolonged toxic effects. In the present study, surface sterilization of explant was done with 70% ethanol and 40-100mg of $HgCl_2$ in 100ml distilled water. The used of 70% ethanol followed by 0.075-0.1% mercuric chloride for the surface sterilization of various bamboo explants was also reported (Rathore et al. 2009). The surface decontamination in *Isoplexis chalcantha* was reported to be effective in the explant with a solution comprising 0.15% (v/v) mercuric chloride and 1 ml of Tween20 surfactant for a duration of 7 minutes (Molina 2008).

The results for contamination rate, aseptic cultures, bud sprouting and phytotoxicity was observed in 2-4 weeks of inoculation in MS medium. Among the 5 treatments, only treatment V, using fungicide bavistin and antibiotics like streptocycline, rifampicin and ciprofloxacin were very effective to reduce the contamination rate of both species. In treatment I, the contamination percent of *B. wamin* was very high with 98%, which decreases to 36% in treatment V. Similarly, high rate of contamination in treatment I was observed for *D. asper* with 88% which is decreased to 22% in treatment V. The contamination % decreased with increasing treament in both the species and the maximum aseptic culture was observed in treatment V with 64% for *B. wamin* and 77% for *D. asper*. The results for bud sprouting showed a higher percent in treatment I where minimum concentrations of fungicide and antibiotics was used. It showed bud sprouting of 90% in *B. wamin* and 93% in *D. asper* which gradually decreases with increasing treatments V to 65% and 37% respectively. The phytotoxicity percent of the explant in *B. wamin* and *D. asper* was minimum with 9% and 6% in treatment I respectively. Whereas, the percent increased with a maximum of 34% and 40% in both the explants respectively. Overall, treatment I-IV had negative effect in decontamination of explants, whereas treatment V had positive effect as compared to other treatment. The effectiveness and 50% of sterile cultures was reported in bamboo with the used of bavistin in combination with antibiotic like streptopencillin, rifampicin, tetracycline and chloromycetin (Das and Rout 1994). Yasodha et al. (2007) sprayed a 0.1% bavistin to the plants before explant collection, and subsequently rinsing them with the same concentration of bavistin,

streptomycin, and kanamycin, minimized inherent contamination in the cultures. The major goal of explant treatment is to raised aseptic culture by eliminating microbial contamination and micropopagation of healthy plants (Fig. 2). However, standardization for the used of fungicide and antibiotic is also crucial to avoid maximum phytotocicity of the plant tissue.

Table 2. Effect of aseptic treatment on contamination rate, bud sprouting and phytotoxicity of *B. wamin and D. asper*. Values given in this table are means of three replicates (n=3).

Effect of aseptic treatment	Treatment				
	I	II	III	IV	V
Contamination%					
B. wamin	98.67 ± 1.33	92.00 ± 2.31	76.00 ± 2.31	60.00 ± 2.31	36.00 ± 2.31
D. asper	88.00 ± 2.31	76.00 ± 2.31	64.00 ± 2.31	52.00 ± 2.31	22.67 ± 2.67
Aseptic culture%					
B. wamin	1.33 ± 2.31	8.00 ± 2.31	24.00 ±2.31	40.00 ± 2.31	64.00±2.31
D. asper	12.00 ± 2.31	23.33 ± 1.76	36.00 ± 2.31	48.00 ± 2.31	77.33 ± 2.67
Bud sprouting from aseptic culture%					
B. wamin	90.67±1.33	81.33±1.33	73.33±2.67	69.33±3.53	65.33±3.53
D. asper	93.33±1.33	88.00±2.31	78.67±3.53	77.33±2.67	37.33 ± 3.53
Dead brown explants (phytotoxicity) from aseptic culture%					
B. wamin	9.33±1.33	18.67±1.33	26.67±2.67	30.67±3.53	34.67±3.53
D. asper	6.67±1.33	12.00±2.31	21.33±3.53	22.67±2.67	40.00 ± 2.31

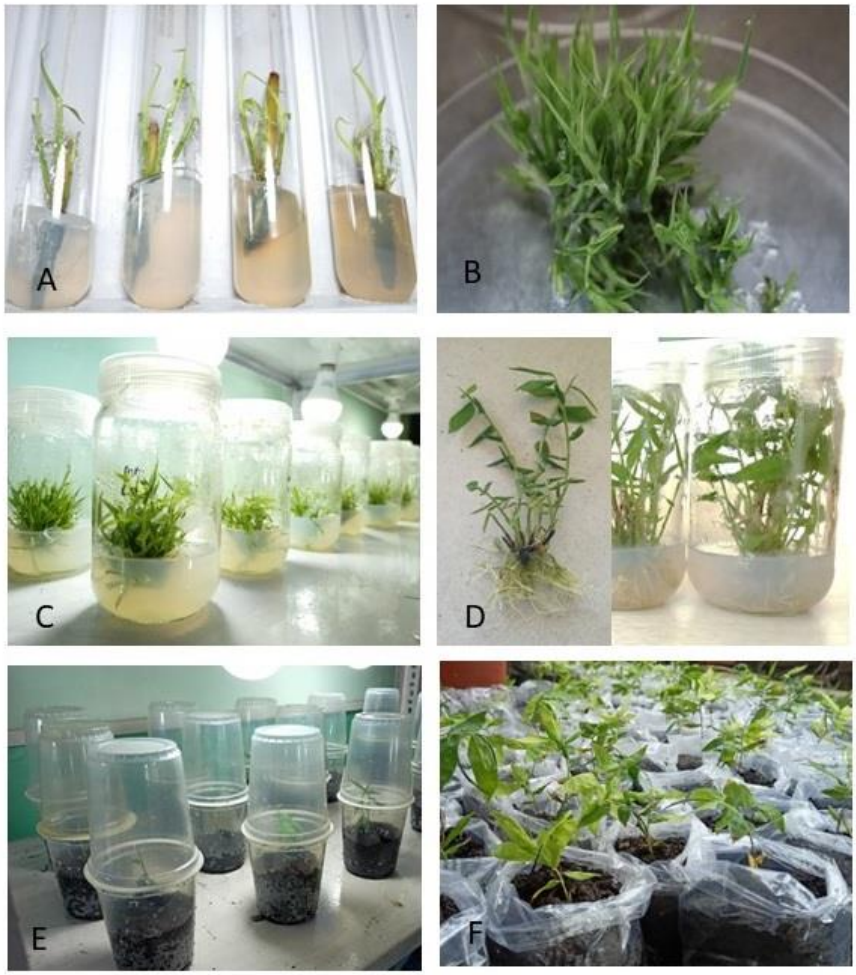

Fig.2 *D. asper*

A. Bud sprouting;

B. Shoot sprouting;

C. Shoot multiplication;

D. Root innitiation;

E-F. Acclimatization.

4. Conclusion

Bamboo stands as a vital natural resource in tropical and sub-tropical regions, serving diverse commercial purposes. For rapid propagation and species conservation, tissue culture is the best practice. However, the endogenous contamination poses a notable obstacle in bamboo tissue culture for its successful implementation. Through the evaluation of sterilization methods and the analysis of contamination rates for nodal explants of *Bambusa wamin* and *Dendrocalamus asper*, it was found that the treatment protocol involving a combination of fungicides and antibiotics, followed by surface disinfection, effectively reduced contamination rates by 22-36% in both species. Shoot multiplication serves as a rapid mode of propagation through tissue culture, and the developed sterilization protocol plays a critical role in controlling endogenous or latent contamination, a significant bottleneck in bamboo tissue culture. Moving forward, the findings of this study provide valuable insights for improving micropropagation techniques and enhancing the sustainability of bamboo resources, thereby contributing to the conservation and utilization of this important natural resource.

Conflict of Interest

The authors declare there is no conflict of interest

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