RESEARCH ARTICLE

CONTROL OF CONTAMINATION BY MODIFIED STERILIZATION PROCESS USING PLANT PRESERVATIVE MIXTURE IN THE *IN VITRO* CULTURES OF *BAMBUSABALCOOA*

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Abstract: *Bambusa balcooa* was one of the commercially important sustainable bamboo species. Establishment of contamination free cultures is crucial step for the successful micro propagation procedures. The nodal explants of *B. balcooa* were treated with different chemicals and antibiotics. An efficient surface sterilization procedure includes the sequential use of different chemicals like 0.5% NaOCl (Sodium hypochlorite), 0.1 % Mercuric chloride treatment, 0.2% Carbendazim, 70% Ethanol resulting in healthy growth of the plants by eliminating the exogenous contamination. Among the different antibiotics incorporated into the MS media (Murashige and Skoog ,1962), addition of PPM (Plant Preservative Mixture) (0.5 ml/L) was found to be effective in controlling the contaminants like bacterial and fungal growth which reduced to 8.33±1.67%.

Keywords: Antibiotics, Contamination, Micropropagation, Plant Preservative Mixture, Surface sterilization

INTRODUCTION

Bambusabalcooa is a tall, evergreen, and economically important bamboo species belonging to the family Poaceae and subfamily Bambusoidae. It is commonly called female bamboo and can be grown in tropical–subtropical regions around the world. Because of the multifarious uses of bamboo, it is used in construction, fuel, fodder, firewood, furniture, mats, handicrafts, musical instruments, paper and pulp industries, flooring, and corrugated sheets etc., (Gosh, 2008).Bamboo shoots are used in making pickles, soups, salads, etc., as they are rich in proteins, carbohydrates, minerals, and amino acids and majorly with low-fat content (Satya *et al.,* 2010). So, it is commonly called cradle to coffin for its multipurpose usage (FAO, 2010).

Bamboo can be naturally regenerated through seeds and vegetative methods like culm cuttings, rhizomes, branch cuttings, offset cuttings, and the methods of macro-proliferation. But the main disadvantages are bamboo has the characteristic feature of a long flowering cycle, seeds are recalcitrant in nature, extraction and transportation of the vegetatively propagated materials is labor intensive process (Sandhu *et al.,* 2018; Ray and Ali, 2017). Considering the challenges of traditional methods of propagation of bamboo, the promising alternative seems to be *in vitro* micropropagation.

Contamination of plant cultures is still a concern that can lead to losses of entire batches of cultures. In order to prevent bacteria and fungus from during the *in vitro*culture sterilization is essential. Explants were also inhabited by the contaminants which are also found in the internal tissues of the plants. Producing sterile and viable *in vitro* plantlets is necessary to increase the efficiency of plant tissue culture (Kaluzna *et al.*, 2013).

The purpose of this study was to examine the role of different antibiotics in eliminating the contamination of *Bambusabalcooa*.

MATERIALS AND METHODS

Plant material

Single-node segments were collected from the healthy branches of *B. balcooa*from the Forest Research Centre, Mulugu, bamboo plantation. The explants chosen are cut into suitable sizes and leaf sheath was removed and the nodal explants were and brought to the laboratory for surface sterilization.

Culture initiation:

For the eradication or removal of microbes, explants are treated with different chemicals during surface sterilization by following 2 different sterilization methods. Fig 1 represents the treatment 1 surface sterilization process. Whereas, Fig.2 represents the treatment 2 surface sterilization process of *B. balcooa*.

In a Biosafety cabinet, all culture inoculations and aseptic manipulations were performed. The cabinet was cleaned with rectified spirit while the HEPA filter was running. Conical flasks, test tubes, forceps, scalpels, other culture vessels and equipment were all previously sterilized using an autoclave. Using a closed hood and ventilation, the cabinet was exposed to UV radiation for 30 minutes before usage. Forceps and scalpels were further disinfected throughout culture operations by dipping in 70% ethyl alcohol and flaming regularly in between the transfers. During inoculation every time, all the instruments were sterilized and used. Both hands were then

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adequately cleansed and swabbed with 70% ethyl alcohol to guarantee aseptic conditions.

Elimination of contamination by using different Antibiotics

After sterilization the ends of the nodal explants were trimmed and they were inserted vertically into the culture media such that the buds were at the same level as the medium. The basal medium Murashige and Skoog (1962) was used by incorporating different antibiotics antibiotics (500 mg/L) were incorporated into the initiation medium like Ampicillin, Streptomycin, a combination of Ampicillin and Streptoomycin and Plant Preservative Mixture (PPM) (500µl/L).ThepH was adjusted to 5.7 ± 0.1 with 1N NaOH (Sodium hydroxide) or 1N HCl (Hydrochloric acid) poured into culture jars and closed with plastic closures. Explants were cultured in the dark at 16/8 hr light: dark cycle.

Statistical analysis

All the recordings and investigations carried out were represented in the Mean ± Standard Error and determined by one-way ANOVA and the means were compared with Duncan's Multiple Range Test (DMRT) at a significance of $P \leq 0.05$ by using SPSS Software and MS Excel.

RESULTS

Surface sterilization

Explants are prone to attack by different kinds of microbes like bacteria and fungi which contaminate the culture (Mekonnen *et al.,* 2013).After the collection of explants, they are thoroughly washed with different disinfectants followed by water wash. In the present study, during the first 7 days of culture, just as the buds began to expand and the sheaths began to separate, contamination was noticed. This indicates that the contaminants were trapped within the sheaths that surround the bud and were unnoticed by the disinfection processes. To get rid of the contamination to the explants, they were treated with disinfectants like tween 20, mercuric chloride, sodium hypochlorite, 70% ethanol, and carbendazim but it could not eliminate the contaminants totally but was effective in reducing the rate of contaminants like bacterial and fungal infection.

In the present study, the following sterilization process was successful in establishing the culture (Fig.2). After collecting the explants, they were trimmed to the required length and placed under running tap water for 5 minutes. after that these explants were treated with a 1% tween 20 solution for about 10 minutes and followed by water wash with distilled water 3 times. Explants were then treated with 0.5% NaOCl for 10 minutes followed by distilled water wash 2 times. After that explants were treated with 0.1% mercuric chloride for about 4 minutes and then washed with distilled water wash 2 times. The explants were then treated with 0.2% carbendazim with 600 mg/L streptomycin sulphate and ampicillin sodium salt for 1 hr 30 min followed by washing with autoclaved distilled water wash for 2-3 times. Then the explants were taken to the biosafety cabinet and treated with 70% Ethanol for 45 seconds followed by autoclaved distilled water wash for 2 times. The explants were then placed on the filter paper and the ends were trimmed with a scalpel blade and inoculated under a spirit lamp.

Elimination of contamination by using different Antibiotics

Contamination was observed very early in culture, usually within the first week(Fig.3A, B,C). Although it was reduced through the inclusion of antibiotics like ampicillin, streptomycin, and Plant Preservative Mixture. Among the different antibiotics, PPM is found to be effective in eradicating the amount of contamination to a level of 8.33 %, followed by a combination of ampicillin and streptomycin sulphate (35%). Whereas, treatment without the addition of antibiotics showed a higher contamination percentage i.e., 98.33% (Table1).

DISCUSSION

Surface sterilization

During the first stages of initial cultures the explants are prone to severe contamination. The fungal contaminats were easily detected by the spore/ fruiting bodies surrounding the explants and also the medium. In the present study to get rid of the contamination to the explants, they were treated with disinfectants like tween 20, mercuric chloride, sodium hypochlorite, 70% ethanol, and carbendazim but it could not eliminate the contaminants totally but was effective in reducing the rate of contaminants like bacterial and fungal infection.

Earlier works reported different surface sterilization procedures i.e., nodal explants of *Bambusabalcooa* with tween 20 solution (Choudhary *et al.,* 2017; Suwal *et al.,* 2021; Sharma and Sarma *et al.,* 2011) and Bavistin in the range of 0.1% (Choudhary *et al.,* 2017; Thapa *et al.,* 2018; Rajput *et al.,* 2020), 0.2% (Das and Pal*et al.,* 2005; Bhadrawale*et al.,* 2017). In our present study 0.2% carbendazim solution was found to be effective in reducing the rate of contamination. Similarly, higher concentrations of Bavistin in the range of 0.5% (Sharma and Sarma*et al.,* 2011; Pratibha and Sarma*et al*., 2013; Barman, 2021); 2% (Suwal*et al.,* 2021) were found to reduce the contamination.

Sodium hypochlorite solution in the range of 0.05 to 10% (v/v) was also usedbyseveral authors like Bhadrawale *et al.,* 2017; Mishra *et al.,* 2008; Das and Pal*et al*.,2005.

Mercuric chloride was used to disinfect the explants of *Bambusawamin* and *Dendrocalamusfarinosus* (Arshad *et al.,* 2005; Hu *et al.,* 2011); *Dendrocalamusaper* species (Arya *et al*., 2008) *Clinacanthus nutans* (Hashim *et al.,* 2021).

Elimination of contamination by using different Antibiotics

The present study demonstrated that treatment with PPM is required for the successful establishment of bamboo in vitro cultures from internal contamination as it does not eliminate the bacteria by the usual surfacesterilization method. In earlier research, endophytic bacteria were also treated with antibiotics like Streptomycin is also found to be a better antibiotic by Leifert*et al.,* 1989. Similarly, Nadha*et al.,* 2012 reported the use of streptomycin sulphate in the media as antibiotic resistance to *Guadua angustifolia* and found that the addition of 15µg/mL was found to be effective in eliminating the bacterial contamination but, it inhibited the shoot growth. This might be because of the prevention of protein synthesis not only in microbial cells which leads to the death of the organism. But it may also be found to inhibit protein synthesis in chloroplasts and mitochondria in plant tissues. Khan *et al.,* 2018 also reported that ampicillin is found to be the least effective antibiotic in *Fagoniaindica.*

PPM is a heat-stable biocide that is efficient against a variety of prevalent *in vitro* contaminants like bacterial and fungus (Guri and Patel, 1998; Niedz, 1998;Kushnarenko *et al.,* 2022). PPM(2mg/L) reduced the contamination in *Guadua angustifolia* (Jimenez *et al.,* 2006). Similarly, Khare*et al.,* 2021 used a Plant Preservative Mixture (500 µl/L) in *Dendrocalamusstrictus* and found to be effective against contamination.In the present study the explants treated with PPM improved the survival percentage and decreased the contamination percentage to 8.3% compared to 98.33% in control. (Table 1).

Table 1. Elimination of contamination by using different Antibiotics

Antibiotic Concentration (500mg/L)	% of Contamination
Control	98.33^{a}±1.67
Ampicillin	61.67^{b}±4.41
Streptomycin	50.0^{c}±2.89
Amp + Strp	35.0^{d}±2.89
PPM *	8.33^{e}±1.67

Data are represented as Mean ± Standard Error. Mean values followed by different superscripts in the same column are significantly different from each other (P<0.05, Duncan's Multiple Range Test). PPM*: Plant Preservative Mixture (500µl/L).

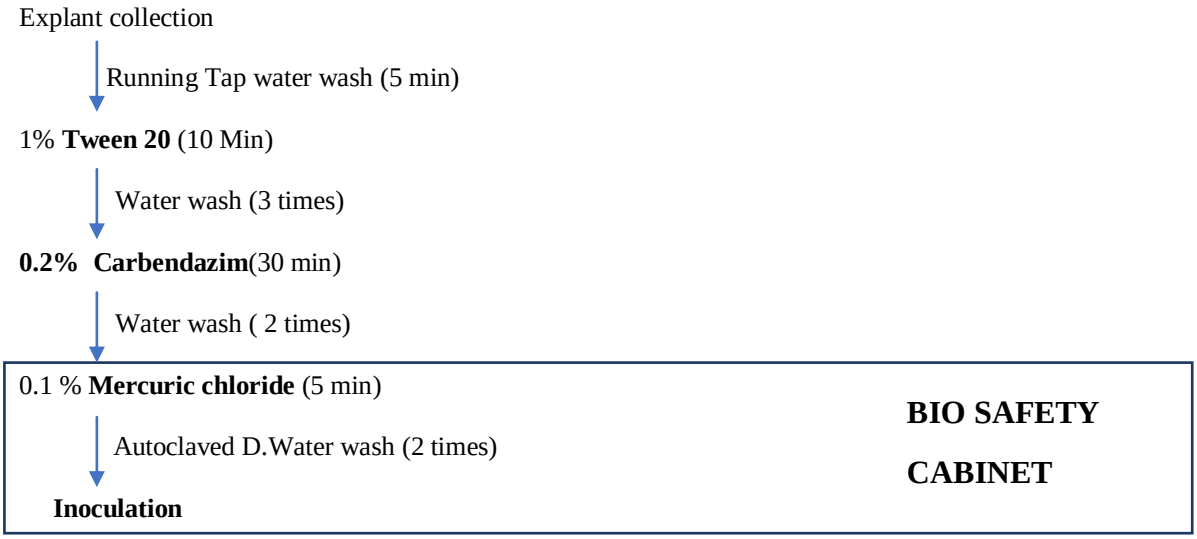
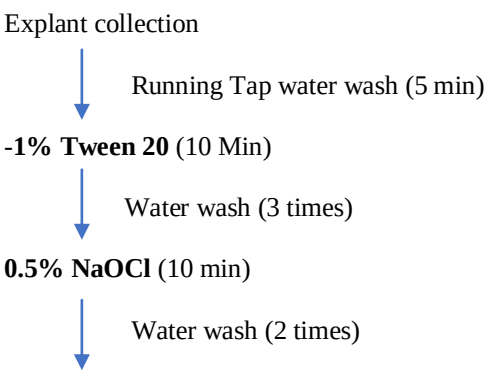


Fig. 1. Surface sterilization process (treatment 1) to explants.



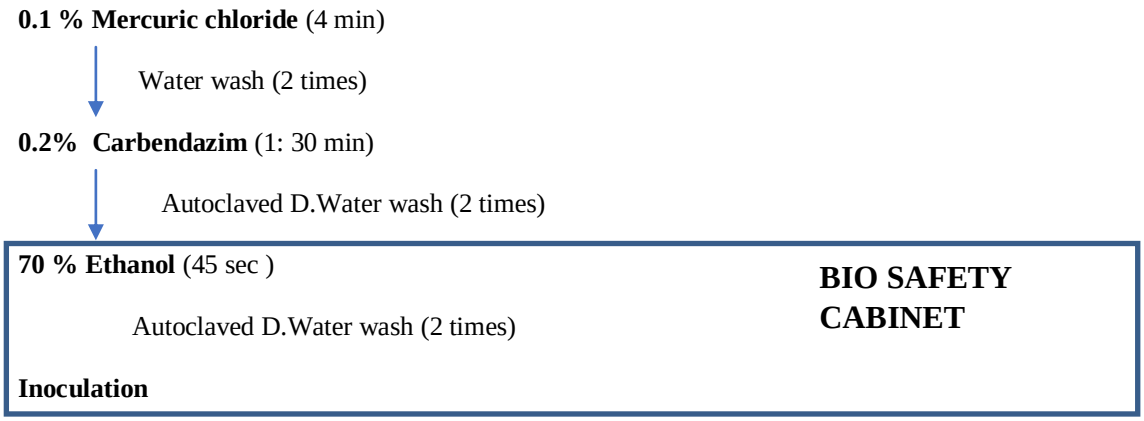


Fig. 2. Surface sterilization process (treatment 1) to explants.

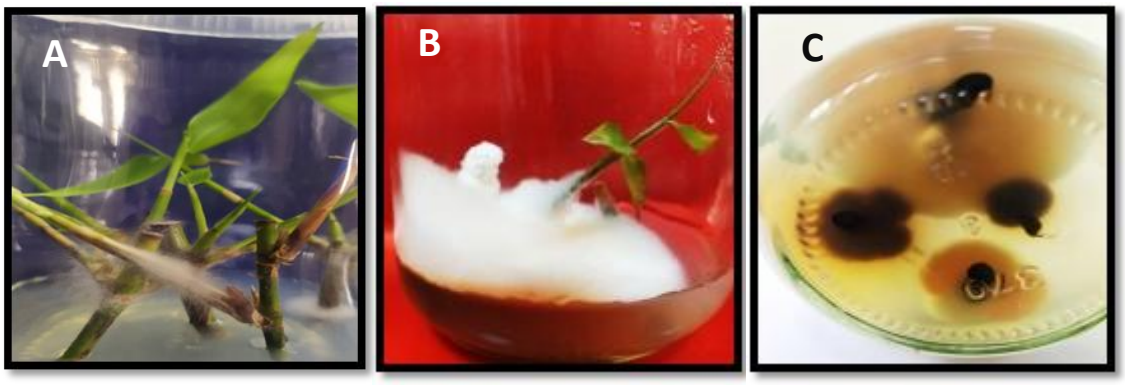


Fig. 3. Contamination of nodal explants of *Bambusa balcooa*. (A, B) Fungal contamination of nodal explants (C) Bacterial contamination of nodal explants.

CONCLUSION

Contamination of cultures with bacteria and fungus is the major impediment for the invitro culture of plants. Disinfection of nodal explants with tween 20, mercuric chloride, sodium hypochlorite, 70% ethanol, and carbendazim was carried out whereas, the use of PPM (Plant Preservative Mixture) (500 µl/L) resulted in reduction in the level of contamination.

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