

Research Article

Evaluation of sterilants on the establishment of *in vitro* regeneration protocol in *Litsea cubeba* (Lour.) Pers.

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How to CiteBui, S. *et al.* (2025). Evaluation of sterilants on the establishment of *in vitro* regeneration protocol in *Litsea cubeba* (Lour.) Pers. *Journal of Applied and Natural Science*, 17(3), 1240 - 1244. <https://doi.org/10.31018/jans.v17i3.6790>**Abstract**

Litsea cubeba holds significant commercial value due to its essential oils and pharmacological properties. Establishing an effective *in vitro* culture system is crucial for mass propagation and conservation of this species. However, microbial contaminants present a common challenge, often hindering the establishment of *in vitro* cultures. The present study was conducted to establish and maintain *in vitro* cultures of *L. cubeba*. The sterilants, viz., mercuric chloride (HgCl_2) and sodium hypochlorite (NaOCl), were evaluated at different concentrations (0.1, 0.2%) and exposure times (3, 5, 7 minutes for HgCl_2 ; 10, 15, 20 minutes for NaOCl) to assess their contamination and survival rates. Among all the treatments tested, treatment T11 (T11) with 0.2% NaOCl for 15 minutes was found to be the most effective, with a survival rate of 97.30% and a contamination rate of 6.76%. The study revealed that NaOCl when applied at optimized concentrations, effectively reduced microbial contamination while maintaining higher explant viability. In contrast, although HgCl_2 demonstrated strong antimicrobial action, it resulted in increased tissue damage and lower survival due to its phytotoxic nature. Extended exposure and higher concentrations of both sterilants significantly affected explant viability and inhibited shoot regeneration. These findings suggest that NaOCl is a more suitable and environment friendly alternative for sterilization in *L. cubeba* culture establishment. The study contributes toward developing a standardized sterilization protocol for efficient micropropagation and conservation of this valuable species.

Keywords: *Litsea cubeba*, sodium hypochlorite, surface sterilization, sterilant, tissue culture**INTRODUCTION**

Litsea cubeba is widely recognized for its essential oils, sesquiterpenoids, flavonoids, lignans, and various secondary metabolites. The compounds are not only valuable for their commercial applications in medicines, insecticides, perfumes, flavours, and colognes, but also for their potential pharmacological activities, including antipyretic, analgesic, antidiarrheal, antimicrobial, anti-inflammatory, antioxidant, and hepatoprotective effects (Kamle *et al.*, 2020; Bui *et al.*, 2024). The *L. cubeba* seeds tend to form long-lived seed reserves (Chen *et al.*, 2013); hence, plant tissue culture provides a suitable alternative to overcome this challenge.

Plant tissue culture is a key technique in modern bio-

technology. It allows for the clonal propagation of elite germplasm, which helps conserve threatened species and promotes sustainable production of secondary metabolites under controlled conditions (Tomaszewska-Sowa *et al.*, 2015). One of the important requirements for successfully establishing *in vitro* cultures is effectively sterilizing explants.

The success of surface sterilization can significantly impact the establishment and growth of *in vitro* cultures, as contamination from both epiphytic and endophytic microorganisms can hinder plantlet development and lead to experiment failure (Gammoudi *et al.*, 2022). Optimizing the sterilization process for each plant species and explant type is crucial, as factors such as explant source, age, and physiological state can influence

the effectiveness of the sterilization treatment (Kuppusamy *et al.*, 2019). Surface sterilization is a critical and often challenging step in plant tissue culture, particularly for woody species such as *L. cubeba*, where culture establishment is hindered by phenolic leaching and microbial contamination (Garg *et al.*, 2014; Luong *et al.*, 2025).

Researchers often need to strike a balance between effectively eliminating contaminants and plant tissues during the sterilization process, which is one of the major challenges in starting *in vitro* cultures. The duration of exposure to sterilizing agents, the concentration of chemicals used, and the sequence of treatments can all be adjusted to achieve optimal results, additionally, the use of antioxidants or pretreatments can help reduce oxidative browning and phenolic exudation, which are common problems in Woody and aromatic species and help improve explant survival rates following surface sterilization (Kilic and Uysal, 2023; Mao *et al.*, 2000; Parmadi *et al.*, 2023; Shah *et al.*, 2013). Chemical sterilant such as mercuric chloride (HgCl_2) and sodium hypochlorite (NaOCl) are commonly used because they have broad-spectrum antimicrobial properties. However, their effectiveness can vary based on exposure time, tissue type, and plant species (Kuppusamy *et al.*, 2019; Bhat *et al.*, 2022). NaOCl is often preferred because it is safer for the environment and less toxic to plant tissues compared to HgCl_2 , which can harm plants during long exposures (Gu *et al.*, 2022; Kumar *et al.*, 2023).

Despite these advancements, reports specifically focusing on optimized sterilization of *Litsea cubeba* remained limited. Therefore, the present study was conducted to optimize the sterilization protocol for *Litsea cubeba*, with the primary objectives of achieving contamination-free cultures and maintaining high explant survival rates.

MATERIALS AND METHODS

Young branches were collected from 1 to 2-year-old trees of *L. cubeba*. After removing the leaves, and shoot tip, node segments were used as explants. These nodal segments were put under running tap water for 30 min and then fungicide treatment was given using Bavistin (0.1%) for time duration (30 minutes), followed by a 15-minute wash with 2 drops of Tween 80 and then washed with distilled water. Further washing was done under laminar air flow, where explants were treated with various concentrations (0.1%-0.2%) and exposure time of sterilizing agents (HgCl_2 (3,5,7 min), NaOCl (10, 15, 20 min). After each treatment, explants were washed with autoclaved/ sterile distilled water 3-4 times to remove any residues left by the sterilant. The explants were cut into appropriate sizes (3-5cm) and surface-dried before being transferred to Murashige and

Skoog (1962) medium with 3% sucrose, 0.8% agar, and 0.5 mg/L BAP+0.2 mg/L NAA. 100mg/L Ascorbic acid was used to maintain the phenolic leaching of the explant following Mao *et al.*, 2000. Media pH was adjusted to 5.8 using 1N NaOH or 1N HCl. The media was sterilized by autoclaving at 1.06kgcm^{-2} pressure and 121°C temperature for 20 minutes. All the cultures were maintained at $24\pm 2^\circ\text{C}$ under a 16h photoperiod with a photosynthetic photon flux (PPF) density of $50\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ provided by white fluorescent lamps (Philips, India).

The data recorded included the survived and contaminated cultures established after 4 weeks of culturing. The data were converted into percentages and the effective sterilization protocol was found by observing the survival rates and the level of contamination formed during the different sterilization treatments.

Ethics statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Statistical analysis

The data were analyzed statistically using SPSS ver. 10 (SPSS Inc., Chicago, USA). The significance of differences among means was determined using Duncan's multiple range test at $p = 0.05$. The results were expressed as the mean $\pm$ S.E. of three repeated experiments.

RESULTS AND DISCUSSION

Effect of various concentrations of mercuric chloride on sterilization

Two different concentrations of mercuric chloride (0.1 and 0.2) were used in this study, subjected to different time intervals (3, 5, 7min). The highest contamination rate was found in treatment 1(T_1) with 0.1% HgCl_2 (73%), where the explants were subjected to 3 min washing, and the least contamination rate was observed in treatment 6 (T_6) with 0.2% HgCl_2 (25%), where the explants were subjected to 7 min washing. The contamination rate decreased with an increase in a time interval, but caused severe oxidative browning and necrosis. In contrast, the survivability rate of explants depended on the exposure time and mercuric chloride concentration. The highest survivability rate was observed in treatment 3 (T_3) with 0.1 % HgCl_2 for 7mins (39.58%), followed by treatment 2 (T_2) with 0.1 % HgCl_2 (23.96%), and the least survivability rate was observed in treatment 6 (T_6) with 0.2% HgCl_2 for 7 mins (2.26%) (Table 1). The explant survival rate of sterilization treatments with HgCl_2 was less compared to the treatment with NaOCl (Table 1). This decline in the survivability rate may be due to the prolonged exposure (>5

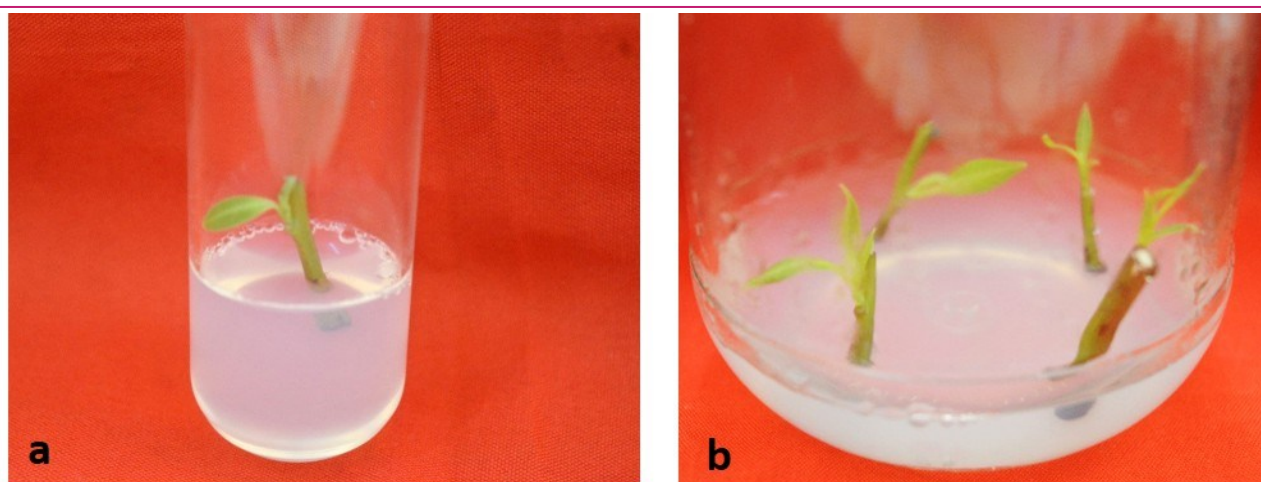


Fig.1. *In vitro* cultures of *Litsea cubeba* in MS fortified with 0.5 mg/L BAP+ 0.2 mg/L NAA. These cultures were established and maintained using treatment 11(T₁₁) with 0.2% sodium hypochlorite for 15 minutes, (a) 15-day-old culture & (b) 30-day-old culture

Table 1. Effect of sterilant (HgCl₂ and NaOCl₂) on *in vitro* culture establishment of *L. cubeba* in MS medium supplemented with 0.5mg/L BAP + 0.2mg/L NAA + 100mg/L Ascorbic acid and 3% sucrose(w/v).

Treatments	Sterilant	Concentration of HgCl ₂ /NaOCl (w/v)	Exposure time (minutes)	Contamination rate (%)	Survival rate (%)
T ₁	HgCl ₂	0.1	3	73.61±0.54 ^f	10.59±0.61 ^b
T ₂		0.1	5	69.79±0.37 ^e	23.96±0.57 ^d
T ₃		0.1	7	31.94±0.28 ^c	39.58±0.39 ^e
T ₄		0.2	3	41.15±0.28 ^d	21.00±0.53 ^c
T ₅		0.2	5	28.82±0.34 ^b	10.42±0.66 ^b
T ₆		0.2	7	25±0.50 ^a	2.26±0.70 ^a
T ₇	NaOCl	0.1	10	93.92±0.70 ^f	11.11±0.54 ^a
T ₈		0.1	15	73.09±0.66 ^e	21.00±0.46 ^b
T ₉		0.1	20	61.28±0.53 ^d	31.77±0.70 ^d
T ₁₀		0.2	10	41.84±0.17 ^c	49.82±0.39 ^e
T ₁₁		0.2	15	6.76±0.42 ^b	97.30±0.56 ^f
T ₁₂		0.2	20	4.34±0.53 ^a	27.77±0.54 ^c

minutes) of explants to HgCl₂. The extended exposure to HgCl₂ has been associated with phytotoxic effects, including browning and death of nodal explants of *Eucalyptus* hybrid clones (Kuppuswamy *et al.* 2019; Mosoh *et al.*, 2023). Different researchers have also used HgCl₂ to achieve aseptic cultures. Fang *et al.* (2012) used 0.1% HgCl₂ for 10 min on nodal explants of *L. cubeba* to achieve a contamination rate of 41.0% and a 15.0% mortality rate. Similarly, 0.5% HgCl₂ for 5 minutes was found to be optimal for obtaining sterile *in vitro* cultures using nodal explants of *L. cubeba*, with 90% survival rate (Luong *et al.*, 2025). Soulangue *et al.* (2007) found 0.1% HgCl₂ to be an effective sterilant for *Cinnamomum camphora* and *Cinnamomum verum*. Although these studies suggest that HgCl₂ is an effective disinfectant, its risk of phytotoxicity makes it less efficient in maintaining explant viability.

Effect of various concentrations of sodium hypochlorite on sterilization

In this experiment, two different concentrations of sodium hypochlorite were used, namely 0.1 and 0.2, for different time ranges (10, 15, 20 min). The highest contamination rate (93.92%) was observed in treatment 7 (T₇) with 0.1 % NaOCl for 10 mins, followed by treatment 8 (T₈) with 0.1% NaOCl for 15 mins (73.09%), and the lowest contamination rate (4.34%) was found in treatment 12 (T₁₂) with 0.2% NaOCl for 20 mins. This indicates that a lower concentration of NaOCl was inadequate to control the contamination. The higher concentration of sodium hypochlorite for a longer duration significantly reduced the plant viability. The highest survival rate (97.30%) was observed in treatment 11 (T₁₁) with 0.2% NaOCl for 15 min, followed by treatment 10 (T₁₀) (49.82%) (Table 1). Although in treat-

ment 12 (T₁₂) with 0.2% NaOCl for 20 minutes, the contamination rate was found to be 4.34%, the survival rate was found to be 27.77%, which suggests that a higher concentration of sterilant for a longer duration causes tissue necrosis. The cultures remained aseptic and green with a contamination rate of 6.76% (Fig. 1). Hence, sodium hypochlorite was identified as an effective sterilant with comparatively lower phytotoxicity than mercuric chloride. A similar result was observed in *Gloriosa superba*, where the viability rate declined due to prolonged exposure of sterilant to the explants (Mosoh et al., 2024).

Sodium hypochlorite is the most widely used sterilant in plant tissue culture (Tomaszewska-Sowah et al., 2015). Researchers often prefer NaOCl for its effectiveness and minimal impact on the environment. Huang et al (1998) used 0.4% sodium hypochlorite for 10 minutes to sterilize explants of *Cinnamomum camphora*. Campos & Pais (1996) sterilized seeds of *Persea indica* using a 10% sodium hypochlorite solution for 30 minutes. Tracy et al (2007) used a 10% sodium hypochlorite solution for 15 minutes to sterilize explants of *Lindera melissifolia* (Walt) Blume. Pelegriani et al. (2011) immersed the shoot tips of *Ocotea porosa* (Nees & Martius) in 0.25%(v/v) and 5%(v/v) sodium hypochlorite for 10 mins. Al-Gabbiesh et al. (2014) used 5% sodium hypochlorite for 15 min with a few drops of Tween 80 to sterilize *Laurus nobilis* explants. Rolli et al (2014) surface sterilized nodal segments of *Adansonia digitata* in 70% ethanol for 30 seconds, 2% sodium hypochlorite for 10 minutes, followed by rinsing with distilled water 3-4 times. Mao et al., (2000) used 0.8% (v/v) sodium hypochlorite along with 0.05% (v/v) tween 20 in distilled water for 15 min to sterilize nodal explants of *L. cubeba*. Despite these developments, few reports are focusing on the optimal sterilization of *Litsea cubeba*. Thus, this study assesses the effectiveness of NaOCl compared to HgCl₂ in establishing aseptic cultures of *L. cubeba* nodal explants. The integration of sterilant (NaOCl and Mercuric chloride) with pre-treatments (Bavistin (0.1%) for 30 minutes, followed by a 15-minute wash with 2 drops of Tween 80) was required to maintain explant viability. Phenolic oxidation is a major issue in woody species like *L. cubeba* and often leads to culture failure. The addition of ascorbic acid (100mg/L) to MS medium significantly reduced phenolic leaching.

Conclusion

In the present study, the influence of different sterilization treatments on nodal explants of *Litsea cubeba* was compared, which indicated that treatment 11 (T₁₁) with 0.2% NaOCl for 15 min significantly reduced contamination and maintained *in vitro* cultures of *L. cubeba*, with a contamination rate of 6.76% and a survival rate

of 97.30%. Hence, treatment 11 (T₁₁) with 0.2 % NaOCl for 15 min was found to be best among all the sterilization treatments tested. Treatments with mercuric chloride exhibited a contamination rate as low as 25%(T₆), but the survival rate of 2.26%, which indicated HgCl₂ carries high risks of phytotoxicity in phenol-rich explants such as *L. cubeba*. The study indicated that sodium hypochlorite (NaOCl) was a more effective and safer sterilizing agent than mercuric chloride (HgCl₂) for establishing and maintaining *in vitro* cultures of *L. cubeba*. Unlike earlier studies that only used HgCl₂, the present study indicates that NaOCl provided a safer, more sustainable option, especially for the sterilization of *L. cubeba* nodal explants. Additionally, using ascorbic acid as pre-treatment as well as in the MS culture medium helped in reducing oxidative stress caused by phenolic compounds. The results offer a standardized, reliable, and low-toxicity sterilization method. This significantly aids in micropropagation, conservation, and commercial farming of this valuable species. Future research can expand on these findings to improve shoot growth and rooting stages, as well as boost secondary metabolites production in controlled *in vitro* conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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