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Optimizing an Appropriate Sterilization Procedure to Control Microbial Contamination during *In vitro* Culture of *Piper longum* L.

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Abstract

Piper longum L., a unisexual perennial climber, is well-known for its high therapeutic potential, but is becoming threatened due to overharvest. The bioactive compounds, responsible for its medicinal properties, are also considered a favorable niche for microorganisms. The persistent contamination caused by these endophytes is the major challenge in micro-propagation. The present study aimed to develop an optimized protocol to reduce microbial contamination by maintaining a high level of regeneration capacity of the cultured explants. For this purpose, eight types of surface sterilization methods and four different antimicrobial agents were used in callus induction media. Among various surface sterilization methods, treatment T_3 showed the minimum level of contamination with a comparatively higher level of explant survivability. In case of antimicrobial agent, the highest effectiveness in controlling contamination with high potential of callus induction capacity was observed in MS media supplemented with 3.0 mg/l 2,4-D and 25 mg/l carbendazim (M_1). In this medium composition the rate of contamination elimination was 82.33% and rate of response in callus induction was 44.67%. Thus, the composition of T_3 sterilization methods and carbendazim as antimicrobial agent can be a suitable procedure to mitigate microbial contamination in *Piper longum* tissue culture by ensuring improved culture viability and regeneration potential.

Introduction

Piper longum L., an aromatic herbaceous plant from the Piperaceae family (Rafiqul et al. 2024), is widely utilized as a culinary spice, and its secondary metabolites have shown

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significant health-promoting effects (Salehi et al. 2019). In Bangladesh, where many people still rely wholly or partially on herbal remedies for primary healthcare (Rahman et al. 2022) and this plant holds significant medicinal importance in this regards. Traditionally, it is used to treat respiratory illnesses, but it also offers a range of other therapeutic benefits, including antioxidant (Natarajan et al. 2006), liver-protective (Christina et al. 2006), anti-inflammatory (Kumar et al. 2009), anticancer (Pradeep and Kuttan 2002) and antimicrobial effects (Ali et al. 2007, Singh and Rai 2013). Due to high demand and widespread use of herbal medicine in the country (Chatterjee and Ghosh 2015), this plant is over harvested from its natural habitats. Unfortunately, its natural reproduction is hampered because of some factors such as low seed viability and germination, poor root development, and habitat degradation (Pradhan 2015, Kanimazi and Sujata 2015). As a consequence, its population in forests is declining, and in some parts of India, it has been listed as a threatened species on the IUCN Red List (Gowthami et al. 2021).

To overcome this situation, identifying an artificial method of reproduction for this plant is essential. Micropropagation stands out as a highly effective approach in this context. Nowadays, tissue culture has gained widespread recognition due to its numerous unparalleled advantages, for example, independence of seasonal variation, rapid production, clones with desired characteristics, preserving endangered plant species and production of novel and genetically improved plants (Omamor et al. 2007).

For a successful tissue culture, a sterile environment is essential (Mug'omba et al. 2012). In spite of strict protocols are properly followed, contamination can still occur because of different endophytes (fungi, bacteria) present in the explants (leaf, nodal segment and root). These microorganisms inhabit the host plant's internal tissue, colonizing inter- and intracellular regions without showing any visible symptoms and causing no harm to the host (Schulz and Boyle 2006). Endophytes and their host plants often engage in a mutual relationship where both are benefitted. In this mutualism, endophytes get shelter, nutrients and access to secondary metabolites from the host while the host plant gains protection against pests and diseases and enhanced growth through the production of phytochemicals and antagonistic compounds by the endophytes (Agusta 2009). Most importantly, the medical plants are considered the best host for endophytes because of the availability of phytochemicals.

The contamination caused by these endophytes in micro-propagation is a major challenge (Altan et al. 2010) as it is tough to eradicate by following conventional surface sterilization procedures. Subsequently, the traditional procedures were diversely modified over the years. Among them, hot-water treatment (HWT) and cold treatment (CT) have shown high effectiveness (Kowalski and Staden 1998). Additionally, synthetic fungicide (carbendazim) and natural fungicides (turmeric, neem and garlic extract) have also exhibited immense effect on the sterilization (Samarakoon et al. 2019, Ramesh et al. 2009). These substances are traditionally used either with the nutrient media or during explant sterilization.

In this context, the present study was conducted to identify the most effective sterilization technique for *Piper* species that ensures tissue integrity while maximizing the success rate of explant survivability.

Materials and Methods

The selected plant (*Piper longum*) was collected from different locations of the Barishal division and maintained in the Net house, University of Barishal. Taxonomic authentication was performed following the methodology described in the volume 9 of *Encyclopedia of Flora and Fauna of Bangladesh* (Ahmed et al. 2009). The explants were collected from the mature leaves of the developed plants grown in net house and subjected to *in vitro* culture.

Surface sterilization of the collected leaf and explants was performed in two different steps i.e. outside the laminar air flow and inside the laminar air flow. In every steps various surface sterilization techniques were applied. The procedure used for surface sterilization process is described in Table 1.

Table 1. Different procedure of surface sterilization.

Treatment	Site of sterilization	Procedure
T1	Outside the laminar airflow	Mature leaves were gently washed by tap water and then soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 0.1% $HgCl_2$ for 6 min. The explants were then kept in Tween 20 for 1 minute. Finally the explants were rinsing three times with sterilized distilled water.
T2	Outside the laminar airflow	Mature leaves were gently washed by tap water and then soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 1% Dettol for 5 min, then placed in 70% ethanol for another 0.5 minute. The explants were then kept in 0.5% $HgCl_2$ for 2 min. Finally the explants were rinsing three times with sterilized distilled water.
T3	Outside the laminar airflow	Mature leaf was gently washed by tap water and then soaked in Tween 20 for 30 min. The leaves were then soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 0.1% $HgCl_2$ for 3 min. The explants were then kept in 70% ethanol for another 1 minute. Finally the explants were rinsing three times with sterilized distilled water.
T4	Outside the laminar air flow	Mature leaf was gently washed by tap water and then soaked in Tween 20 for 30 min. The leaves were then soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 70% ethanol for 5 min. The explants were then kept in NaOCl for another 5 min. Finally the explants were rinsing three times with sterilized distilled water.
T5	Outside the laminar airflow	Mature leaf was gently washed by tap water and then soaked in Tween 20 for 10 min. The leaves were then soaked in distilled water for 5 min and transferred into laminar air flow.

	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 1% fungicide for 25 min and then placed in 70% ethanol for 1 minute. The explants were then kept in 0.1% $HgCl_2$ for another 5 min. Finally the explants were rinsing three times with sterilized distilled water.
T6	Outside the laminar airflow	Mature leaf was gently washed by tap water and then soaked in Tween 20 for 15 min. The leaves were then soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 1% fungicide for 30 min and then placed in 100% ethanol for 0.5 minute. The explants were then kept in 0.1% $HgCl_2$ for another 2 min. Finally the explants were rinsing three times with sterilized distilled water.
T7	Outside the laminar airflow	Mature leaf was gently washed by tap water and then soaked in 70% ethanol for 1 minute. The leaves were then soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 0.1% $HgCl_2$ for 5 min, then placed in 20% NaOCl for 15 min. The explants were then kept in 70% ethanol for another 1 minute. Finally the explants were rinsing three times with sterilized distilled water.
T8	Outside the laminar airflow	Mature leaf was gently washed by tap water, then soaked in 5% Tween 20 for 15 min. After that the leaves were kept in 20% NaOCl for 15 min, Finally, the leaf were soaked in distilled water for 5 min and transferred into laminar air flow.
	Inside the laminar airflow	Cutting explants to around 1 cm and soaked in 0.1% $HgCl_2$ for 5 min. The explants were then kept in 70% ethanol for another 1 minute. Finally the explants were rinsing three times with sterilized distilled water.

Table 2. Media combination with different antimicrobial agents.

Media	Combination
M_0	MS+ 2 4 D (3mg/l)
M_1	MS+ 2 4 D (3mg/l) + 25mg/l Carbendazim
M_2	MS+ 2 4 D (3mg/l) + 50mg/l Carbendazim
M_3	MS+ 2 4 D (3mg/l) + 100mg/l Carbendazim
M_4	MS+ 2 4 D (3mg/l) + 0.5 g/l Turmeric extract
M_5	MS+ 2 4 D (3mg/l) + 1.0 g/l Turmeric extract
M_6	MS+ 2 4 D (3mg/l) + 2.0 g/l Turmeric extract
M_7	MS+ 2 4 D (3mg/l) + 0.5 g/l Garlic extract
M_8	MS+ 2 4 D (3mg/l) + 1.0 g/l Garlic extract
M_9	MS+ 2 4 D (3mg/l) + 2.0 g/l Garlic extract
M_{10}	MS+ 2 4 D (3mg/l) + 0.5 g/l Neem extract
M_{11}	MS+ 2 4 D (3mg/l) + 1.0 g/l Neem extract
M_{12}	MS+ 2 4 D (3mg/l) + 2.0 g/l Neem extract

The surface sterilized explants were then inoculated on MS media supplanted with different concentration of 2,4-D for *in vitro* regeneration. For the preparation of MS media 4.4 gm/l MS powder, 3% (w/v) sucrose as a carbon source, and 0.8% (w/v) agar were

used. To eliminate the microbial contamination different natural and synthetic antimicrobial agent was applied with callus induction media (Table 2). A total of 100 explants for each treatment were inoculated in 20 different culture vessels. Three replications of each treatment were applied.

Contaminated data were collected after 3 weeks of inoculation in culture media. The green colored non contaminated explants were separated and counted as viable explants. For callogenesis, the explants were kept in the respective media up to 6 weeks.

The collected data were statistically analyzed to determine the significance of the experimental results. Analysis of variance (ANOVA) was conducted for all traits using the Least Significant Difference (LSD) test. The statistical analyses were carried out using STATISTIX 10 software. Differences among treatment means were considered significant based on the LSD test at a probability level of $p \leq 0.05$, as outlined by Gomez and Gomez (1984).

Results and Discussion

This study aimed to develop a suitable surface sterilization protocol for leaf explants of *Piper longum* L. by ensuring the highest viability. This experiment was carried out in two different phases. At the initial stage, it involved evaluating multiple sterilization treatments and finally assessing the efficacy of various antimicrobial agents towards the performance in minimizing contamination.

In the surface sterilization part, T_8 showed the best performance in terms of controlling contamination where, the least contamination (9.333%) was recorded. Although it performed best in eradicating contamination but it showed an adverse effect on the viability of the explant. Here, the explant became faded in color, which indicated the loss of viability of that explant. The second best performance was recorded from treatment T_3 where the rate of contamination was 13.667%. However, the explant viability rate was higher (42%) in this procedure. As explant survival rate is an important part for *in vitro* regeneration, in this context, considering the rate of contamination and viability T_3 was considered as best performing treatment for surface sterilization methods for the leaf explant of *Piper longum* L. The overall responses of different antimicrobial agents are shown in Table 3.

In the second phase of this experiment, explants were cultured on MS media supplemented with different concentration of 2,4-D following the surface sterilization procedure of T_3. In this step different types of natural (extracts of ginger, turmeric and neem) and synthetic (carbendazim at various concentrations) antimicrobial agent was applied in callus induction media to select the suitable antimicrobial agent for *Piper* towards eliminate the microbial contamination as well as enhance the viability of cultured explants.

Table 3. Effects of different surface sterilization protocols.

Treatments	% of response towards		
	Contamination	Non-contamination	Survivability of explants
T_1	49.000 c	51.000 b	8.000 bc
T_2	71.667 b	28.333 c	2.667 cde
T_3	13.667 c	86.333 b	42.000 a
T_4	97.000 a	3.000 d	2.333 de
T_5	74.333 b	25.667 c	4.000 bcde
T_6	93.333 a	6.667 d	0.667 e
T_7	53.000 c	47.000 b	6.333 bcd
T_8	9.333 d	90.667 a	8.667 b
Mean	61.417	38.583	9.3333
CV (%)	15.40	24.52	30.07
F Value	27.82	27.82	69.41
SEM	7.7236	7.7236	2.2913
LSD (0.05)	16.566	16.566	4.9143
Significance	**	**	**

(** indicates significant at $p \leq 0.01$. Means were calculated from three replicates for each treatment. Values with different letters are significantly different at $p \leq 0.05$, applying the LSD test)

From this experiment, it was observed that MS medium supplemented with 3.0 mg/l 2,4-D and 25 mg/l carbendazim (M_1) showed the best callus induction media that possessing low level of microbial contamination (17.67%) with higher level of explant viability (52%) and callus induction efficacy (44.67%).

Table 4 exhibits the highly variable performances of the different antimicrobial agents. Media M_1 performed the best in terms of viability (52%) and callus induction (44.667%). The overall result in this regards are presented in Table 4 and Fig. 1.

Microbial contamination remains a serious issue in the plant tissue culture especially for the medicinal plants. The probable reason behind this contamination is the presence of different endophytic populations or factors associated with the culture media itself (Onwubiko et al. 2013). To mitigate such microbial contamination, a range of chemical sterilizing agents is typically employed during the surface sterilization of explants. Among these, sodium hypochlorite (NaOCl) is widely used due to its effectiveness and accessibility (Muhammad et al. 2004). Mercuric chloride ($HgCl_2$) is another commonly applied disinfectant, known for its strong antimicrobial properties (Habiba et al. 2002, Molla et al. 2004, Titov et al. 2006). Notably, the combined use of both NaOCl and $HgCl_2$ has also demonstrated enhanced decontamination efficacy in some studies (Rahman et al. 2002, Madhulatha et al. 2004). For instance, Goswami and Handique (2013) reported

Table 4. Effects of different antimicrobial agents supplemented in the media.

Treatments	% of			
	Contaminated explants	Non-contaminated explants	Viable explants	Responsive explants towards callus induction
M_0	43.667 a	56.333 b	33.333 b	20.333 b
M_1	17.667 ab	82.333 ab	52.000 a	44.667 a
M_2	36.000 ab	64.000 ab	1.667 c	0.3333 c
M_3	28.333 b	71.667 a	0.333 c	0.0000 c
M_4	33.333 b	67.667 a	1.667 c	0.0000 c
M_5	32.333 b	67.667 a	2.000 c	0.6667 c
M_6	29.667 b	70.333 a	2.000 c	0.6667 c
M_7	34.333 ab	65.667 ab	2.333 c	1.000 c
M_8	32.000 b	68.000 a	3.000 c	2.0000 c
M_9	29.000 b	71.000 a	1.667 c	0.3333 c
M_{10}	33.667 b	66.333 a	1.667 c	0.6667 c
M_{11}	31.667 b	68.333 a	2.667 c	0.6667 c
M_{12}	29.000 b	71.000 a	2.000c	0.6667 c
Mean	33.128	66.872	8.1795	5.5385
CV (%)	17.52	8.68	30.44	37.11
F Value	1.59	1.59	120.55	119.38
SEM	4.7379	4.7379	2.0332	1.6782
LSD (0.05)	9.7785	9.7785	4.1963	3.4636
Significance	NS	NS	**	**

(** and NS indicate significant at $p \leq 0.01$ and non-significant respectively. Means were calculated from three replicates for each treatment. Values with different letters are significantly different at $p \leq 0.05$, applying the LSD test).

optimal sterilization results for *Musa* species when both agents were applied. In our study, different surface sterilizing agent was used under eight different processes. Among them, surface sterilization with Tween 20, 70% ethanol and 0.1% $HgCl_2$ (T_3) showed the best response towards elimination of microbial contamination.

However, the use of these sterilizing agents can adversely affect the viability of the explants. Improper application, specifically, incorrect concentrations or exposure durations may compromise the physiological integrity of the explants. Abbasi et al. (2016) reported that the failure to optimize the type, concentration, and exposure time of sterilizing chemicals significantly reduced explant viability. Overexposure of $HgCl_2$ may lead to browning and death of explants (Danso et al. 2011). Similarly, elevated concentrations of NaOCl may cause damage to DNA, which ultimately leads to loss of viability (Dukan et al. 1999). Rehman et al. (2023) reported that explants treated with

0.1% $HgCl_2$ for 5 min resulted in a 60% survival rate and 20% contamination, whereas 1.0% $HgCl_2$ treatments significantly reduced the response rate. Mihaljevic et al. 2013, Thompson et al. 2009, Tiwari et al. 2012 also reported a similar effect of the sterilizing agents' concentration and time on the explants' viability and growth. More or less similar type of observation we also faced in our experiments. Even though treatment T_8 was the most effective in reducing microbial contamination in this case, the explant survival rate was rather low.

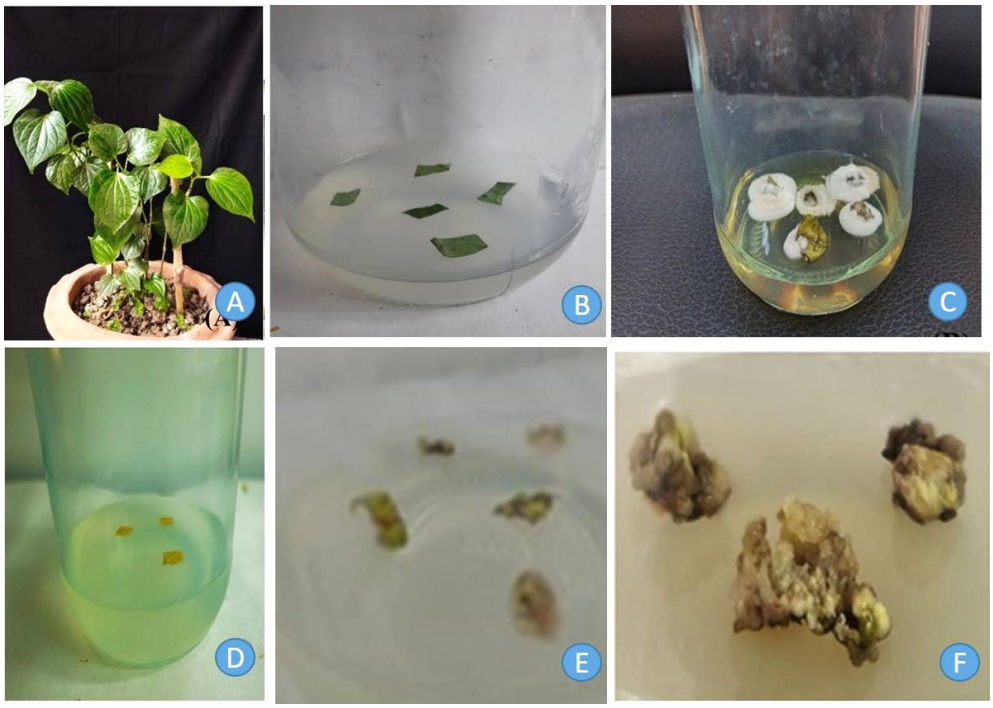

Fig. 1. Different stages of *in vitro* culture: (A) Matured plant, (B) Explant inoculated in culture media, (C) Contaminated explants, (D) Non contaminated but non-viable explants, (E) Callus after 3 weeks of inoculation in MS media supplemented with 3.0 mg/l 2,4-D and 25 mg/l Carbendazim and (F) Same as Fig. (C) in case of 6 weeks after inoculation.

To confirm the reduction of contamination by ensuring the viability of explants, different antimicrobial agents were supplemented with the nutrient media. Among them, carbendazim, when combined with callogenesis media, proved particularly effective, leading to improved explant survival and lower contamination rates. Despite following the surface sterilization protocol, and aseptic conditions, the contamination still appeared in tissue culture (Orlikowska et al. 2016). Since excessive use of chemical sterilants can damage explants, incorporating antimicrobial agents directly into the culture medium offers a viable alternative for combating endophyte-related contamination. Carbendazim

is one such agent that has shown promising results, notably in studies involving purple passion fruit (Prammanee et al. 2011) and Indian Siris (Saeed and Shahzad 2015) but high concentration of carbendazim is phytotoxic to *in vitro* explants, and resulted in the death of almost all treatment cultures. Our result also agrees with this experiment where high concentration of carbendazim (100 mg/l) resulted in the death of almost all treatment cultures.

Contamination by microbes has been a persistent problem for the *in vitro* propagation of *Piper longum*. Another serious consistent of micro-propagation of this plant is the presence of secondary metabolites which cause subsequent browning and necrosis of explants. This study establishes a standardized protocol for surface sterilization of leaf explants from *Piper longum* L. It also identifies the most effective antimicrobial agent to minimize contamination while maintaining a high viability rate. This protocol could be an efficient procedure to eliminate contamination with higher potential for *in vitro* propagation of *Piper longum*. Further research can explore the effectiveness of other natural antimicrobials, not only for *Piper longum*, but also for other *Piper* species.

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