ISSN : 2319-2607 (Print); 2583-0856 (Online) IISUniv.J.Sc.Tech. Vol.12(1), 98-104 (2023)
RESEARCH ARTICLE

Evaluation of the Most Efficient Method for Surface Sterilization of *Azadirachta indica* Leaves for Initiating *in vitro* Cultures

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Abstract

Obtaining microbe-free cultures is a crucial step in the initiation of plant tissue culture. The present study focuses on investigating the different sterilization regimes for leaf explants from *Azadirachta indica* growing under natural environmental conditions, from wild mature trees. In our study, various sterilizing agents ($HgCl_2$, EtOH, NaOCl, and $AgNO_3$) were evaluated at different concentrations and contact durations to determine their effectiveness. Our study indicated that treatment with 0.1% $HgCl_2$ for 5 minutes demonstrated superior results compared to the other agents. Overall, we found that 0.1% $HgCl_2$treatment on mature leaves of *Azadirachta indica* resulted in a 90% survival rate of the explants, with only 10% of the explants experiencing contamination-related death.

Keywords: *Azadirachta indica*, $HgCl_2$, Microbial contamination, Surface sterilization, Plant tissue culture.

Introduction

Azadirachta indica A. Juss., commonly known as Neem, Margosa, and Indian lilac, belongs to the Meliaceae family **(**Koul and Wahab, 2004). Neem is an evergreen tree with distinctive features like a broad crown and an average height of about 25 metres, neem is generally found in tropical woodlands. Neem is often referred to as a "wonder tree" because of its myriad uses **(**Dhaliwal *et al*., 2004**)**. This extremely adaptable tree, which exhibits the capacity to flourish in soils deficient in critical nutrients and exhibits extraordinary resilience to drought, is extremely valuable and widely used (Radwanski, 1977). Even though it is believed that neem originated in the Indian subcontinent and Myanmar, its introduction has helped it spread far, especially in places like Africa, Latin America, Fiji, and several tropical islands (Koul and Wahab, 2004).

A multitude of biological properties have been identified in *Azadirachta indica*, leading to investigations into its potential antibacterial, antiviral, antifungal, molluscicidal, and antihyperglycemic characteristics (Ufele *et al*., 2013; Ezeigwe *et al*., 2015; Abdelhady *et al*., 2015; Ashfaq *et al*., 2016; Sinha *et al*., 2017; Osman Mohamed Ali *et al*., 2017; Altayb *et al*., 2022).

Plant tissue culture plays a vital role in fundamental research in a wide range of fields, such as plant physiology, plant pathology, plant secondary metabolites etc. (Espinosa-Leal *et al*., 2015; Chandran *et al*., 2020; Vidyagina *et al*., 2021; Taalat *et al*., 2021). Plant tissue culture is a technique used to proliferate plant cells, tissues, or organs in a controlled environment from mother plant/explant under aseptic condition **(**Omamor *et al*., 2007). Endophytic bacteria are naturally present within the explants (Hardoim *et al*., 2008). However, additional microbial contamination can arise from improper aseptic handling, unsanitary laboratory settings, or the use of contaminated laboratory equipment. Such contamination is a significant challenge in the development of *in vitro* cultures (Webster *et al*., 2003). These unwanted microorganisms can lead to issues such as increased mortality, stunted growth, tissue necrosis, decreased shoot proliferation, and impaired roots, as they compete with plant tissue cultures for nutrients (Oyebanji *et al*., 2009).

In plant tissue culture, the sterilization of explants remains paramount, given the recurrent challenges posed by microbial contamination. Such contamination frequently hinders the progression of *in vitro* cultures (Zinabu *et al*., 2018) Therefore, the complete elimination of microorganisms becomes imperative for the successful initiation, growth, and development of tissues *in vitro*. Plant tissue culture media, abundant in nutrients and sucrose, provide a favourable environment for the growth of various microorganisms such as bacteria and fungi (Omamor *et al*., 2007). Unfortunately, these contaminants tend to proliferate more rapidly than the cultured tissues themselves, ultimately leading to tissue death. The presence of these competing contaminants

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deprives the explants of essential nutrients, resulting in increased culture mortality (Zinabu *et al*., 2018).

The process of surface sterilization for explants involves immersing them in an appropriate concentration of chemical sterilant or disinfectants for a specified duration, resulting in the establishment of a culture free from contamination. Existing literature has demonstrated the utilization of various disinfectants, including ethanol (EtOH), sodium hypochlorite (NaOCl), mercuric chloride ($HgCl_2$), and silver nitrate ($AgNO_3$).

In a study by (Odutayo *et al*., 2007), bacteria and fungi can be suppressed by a 20-minute NaOCl treatment and a 15-second exposure to 70% ethanol. In a similar way, mercuric chloride also prevents these microbes. Note that NaOCl has potent antibacterial properties as well (Odutayo *et al*., 2007). In another study by (Ashokan *et al*., 2020), Surface sterilization of explants was done using 70% (v/v) of commercially available bleach, Chlorox® (5.25% (w/v) sodium hypochlorite as the active ingredient) for suppression of fungal and bacterial contamination. Most research on explant sterilization in *Azadirachta indica* (neem) has predominantly utilized Mercury chloride but comparative studies is not well documented. The present study aims to determine the optimal method for surface sterilization of leaf explants from *Azadirachta indica*. The study evaluates the efficacy of four sterilizing agents—Mercuric Chloride, Sodium hypochlorite, Ethyl alcohol, and Silver Nitrate—at varying concentrations and exposure times to disinfect these explants for developing a robust protocol for efficient plant tissue culture studies.

Materials and Methods

Plant Material

Neem trees that were at least 15 years old and exhibited disease-free and robust health traits were selected for explants.

Selection of Explant

A preliminary study was conducted for the selection of explants for callus initiation on MS medium. Four explants were tested that include flower, cotyledon, nodal stem segment, leaf (mature and young). It was observed that callus initiation on flower and young leaf was fast and highly reproducible, but cotyledon and nodal stem segments were not much effective in callus production. But for further studies, young green leaf was finalized as an explant because it has an advantage over other explant types as availability all the year round and low contamination as compared to nodal segments. Flower and cotyledons are available for 2-3 months in a year, so they were not selected.

Standardizing of Surface Sterilization of Explants (Disinfection)

For leaf explant disinfection or sterilization, four different sterilizing agents were tested for different concentrations and different exposure times as given in table 1.

Table 1. Different sterilizing agents, their concentrations, and the exposure times tested

These were the combination for testing suitable sterilizing agents for leaf sterilization. 10 explants were used in each combination. For analysis three data parameters were recorded which are (1) explant died due to contamination (%), (2) explant died due to adverse effect of agents (%), (3) live explant (%).

Sterilizing Agent	Concentration (%)	Exposure Time (Minutes)
Mercuric chloride	0.1, 0.5, 1	5, 10, 15
Sodium hypochlorite	5, 10, 15	5, 10, 15
Ethyl alcohol	50, 60, 70	1, 3, 5
Silver nitrate	0.5, 1, 2	5, 10, 15

Explants were first thoroughly washed with tap water. Subsequently, they were rinsed with distilled water containing a drop of the detergent Tween 20 for 15-20 minutes to remove particles adhering to the surface. Then explants were washed with distill water carrying 0.1% carbendazim (bavistin) and streptomycin each for

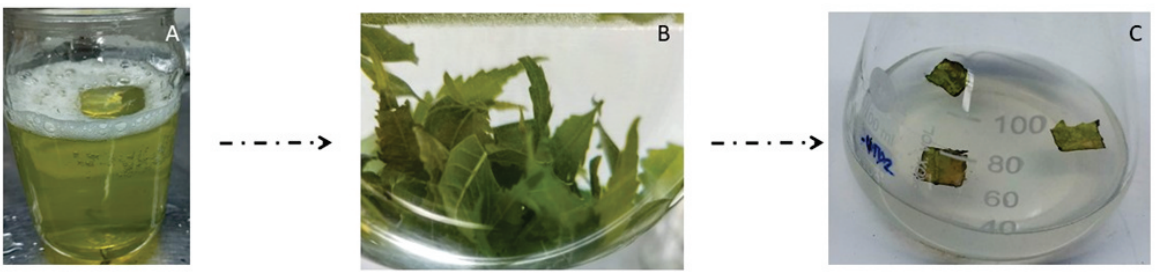

Fig. 1. (A) Treatment of explant with Sterilizing agent, (B) Treated Explant, (C) Explant Inoculated on media

15-20 min. Explants were transferred to a laminar air flow cabinet and surface sterilization with sterilizing agents was performed (Fig. 1).

Statistical Analysis

Experiments were performed with three replicates per treatment. The presented data represent the results of one experiment, which was carried out at least twice. Data was displayed with mean and standard error (SE). IBM SPSS version 22 software was used to analyse the data and One-way analysis of variance (ANOVA) was used to identify differences, and Duncan's multiple range test (DMRT) was used to calculate significant ($P \leq 0.01$) differences between mean values.

Results

A detailed comparison of four sterilizing agents top performing data are showed in Fig. 2 and Table 2 below. Among the sterilant evaluated, $HgCl_2$ at 0.10% concentration for 5 minutes demonstrated the most promising results in terms of explant survival, with a minimal rate of contamination-related death. On the other hand, while $AgNO_3$ at 2% concentration for 10 minutes showed a decent survival rate, it had the highest contamination-related death rate among the sterilant.

Sterilization with $HgCl_2$

Explant were treated with $HgCl_2$ (0.1%, 0.5%, 1%) having different time interval (5, 10 and 15 minutes). It was observed that 0.1% for 5 min have the least contamination rate (10%) and highest live explant (90%) with no death of explant (0%) due to adverse effect of 0.1% $HgCl_2$ as shown in fig. 3.

Sterilization with NaOCl

Explant when treated with different concentration (5%, 10%, 15%) of NaOCl (v/v) solution (4% w/v) with different time duration (5, 10, 15 minutes). It was observed that 5% NaOCl with 15min treatment time was found to be better among other NaOCl combinations with contamination (30%) and live explant (70%) with no death of explant (0%) followed by 15% NaOCl with 15 min treatment having contamination (20%), live explant (63.33%) and dead explant (16.67%) (Fig. 4)

Sterilization with EtOH

Explant when treated with different concentration (50%, 60%, 70%) of EtOH (v/v) with different time duration (1, 3, 5minutes). It was observed that 70% with 3min was better (Fig. 5) performing among other combinations of EtOH with 20% contamination, 56.67% live explant and 23.33% death of explant due to adverse effect of EtOH.

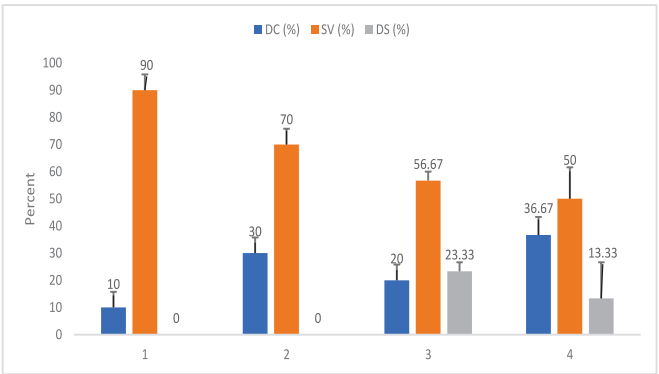

Fig. 2. Comparison of best four sterilizing agent (DC-Death due to contamination, SV-Survival, DS- Death due to overdose of Sterilizing agent (1-$Hgcl_2$ (5 min.), 2-NaOCl (15 min.), 3-EtOH (3 min.), 4-$AgNO_3$, (5 min.)

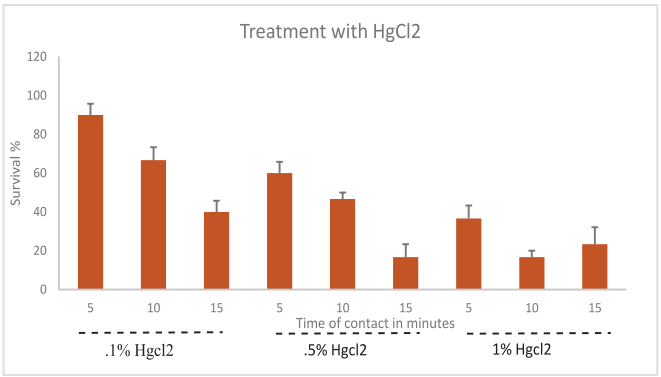

Fig. 3. Survival % of explant after treatment with $HgCl_2$

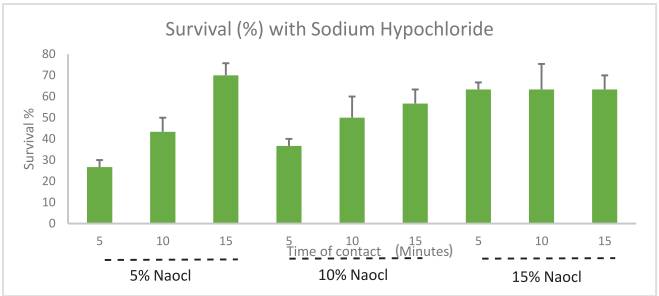

Fig. 4. Survival % of explant after treatment with Sodium Hypochloride

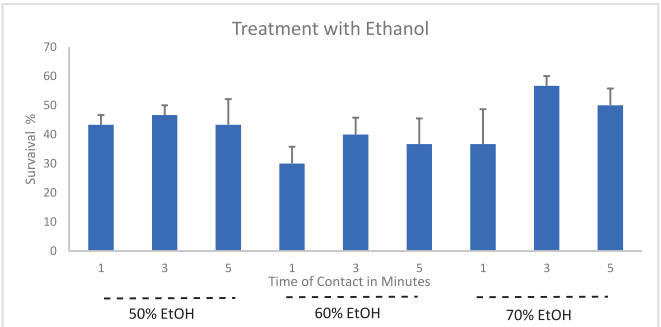

Fig. 5. Survival % of explant after treatment with Ethanol

Table 2: Effect of different sterilizing agents on disinfection of *A. indica* explant

Treatment	Concentration (%)	Time of contact (min)	Explants Dead due to contamination (DC), (%)	Dead due to overdose of sterilant (%)	Survival (SV) (%)
$HgCl_2$	0.1%	5	10±5.77^{a}	0.00±0.00^{d}	90±5.77^{a}
		10	6.67±3.33^{d}	26.67±8.82^{b}	66.67±6.67^{b}
		15	3.33±3.33^{d}	56.67±6.67^{a}	40±5.77^{c}
	0.5%	5	0.00±0.00^{d}	40.00±5.77^{b}	60±5.77^{b}
		10	3.33±0.33^{d}	50±0.00^{b}	46.67±3.33^{c}
		15	0.00±0.00^{d}	83.33±6.67^{a}	16.67±6.67^{d}
	1%	5	3.33±0.33^{d}	60.00±10^{b}	36.67±6.67^{c}
		10	0.00±0.00^{d}	83.33±3.33^{a}	16.67±3.33^{a}
		15	0.00±0.00^{d}	76.67±8.82^{a}	23.33±8.82^{b}
NaOCl	5%	5	66.67±8.82^{b}	6.67±3.33^{d}	26.67±3.33^{c}
		10	50±11.55^{c}	6.67±3.33^{d}	43.33±6.67^{b}
		15	30±5.77^{b}	0.00±0.00^{de}	70±5.77^{a}
	10%	5	56.67±8.82^{b}	6.67±3.33^{d}	36.67±3.33^{d}
		10	43.33±8.82^{b}	3.33±0.33^{de}	50.00±10.00^{b}
		15	43.33±6.67^{b}	0.00±0.00^{e}	56.67±6.67^{b}
	15%	5	33.33±0.33^{c}	3.33±3.33^{d}	63.33±3.33^{a}
		10	36.67±12.02^{c}	0.00±0.00^{e}	63.33±12.02^{a}
		15	20.00±5.77^{d}	16.67±8.82^{c}	63.33±6.67^{a}
EtOH	50%	1	53.33±3.33^{c}	56.67±3.33^{a}	43.33±3.33^{c}
		3	46.67±8.82^{b}	53.33±3.33^{a}	46.67±3.33^{c}
		5	40±5.77^{c}	53.33±8.82^{a}	43.33±8.82^{a}
	60%	1	70±5.77^{a}	0.00±0.00^{d}	30±5.77^{d}
		3	46.67±8.82^{c}	13.33±13.33^{b}	40±5.77^{d}
		5	63.33±8.82^{a}	0.00±0.00^{d}	36.67±8.82^{c}
	70%	1	20.00±5.77^{b}	43.33±16.67^{b}	36.67±12.02^{c}
		3	20.00±5.77^{b}	23.33±3.33^{bc}	56.67±3.33^{a}
		5	13.33±3.33^{c}	36.67±6.67^{b}	50.00±5.77^{a}
$AgNO_3$	0.5%	5	70±5.77^{d}	0.00±0.00^{d}	30±5.77^{c}
		10	50±11.55^{c}	16.67±16.67^{b}	33.33±8.82^{c}
		15	60±11.55^{cd}	0.00±0.00^{d}	40±11.55^{b}
	1%	5	53.33±6.67^{c}	0.00±0.00^{d}	43.33±8.82^{b}
		10	40±5.77^{c}	23.33±12.02^{b}	36.67±12.02^{c}
		15	40±11.55^{c}	16.67±8.82^{a}	43.33±8.82^{b}
	2%	5	40±5.77^{c}	20.00±11.55^{b}	40.00±10.00^{b}
		10	36.67±6.67^{b}	13.33±13.33^{c}	50.00±11.55^{a}
		15	40±11.55^{c}	20.00±20.00^{c}	40.00±11.55^{b}

DMRT; mean followed by different letters differs significantly at p≤0.05; SE- standard error, Number of explant-10, replicate-3

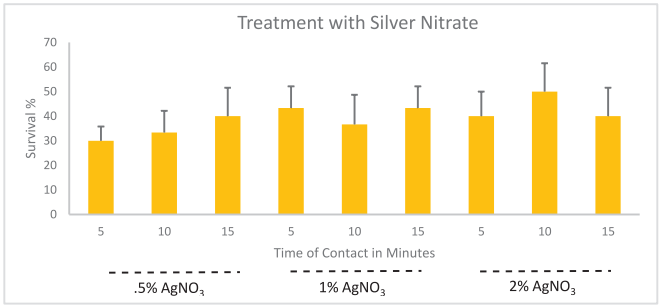

Fig. 6. Survival % of explant after treatment with Silver Nitrate

Sterilization with $AgNO_3$

Explant when treated with different concentration (0.5%, 1%, 2%) of $AgNO_3$ (w/v) with different time duration (5, 10, 15minutes). It was observed that 2% $AgNO_3$ (w/v) with 10min treatment time was performing better (Fig. 6) among all combinations of $AgNO_3$ with 36.67% contamination, 50% live explant, and 13.33% death by adverse effect. It was observed that among all combination of chemical sterilization, explants treated with 0.1% $HgCl_2$ for 5mintues, shows the maximum sterilizing effect with the least contamination rate and necrosis along with the highest live explant.

Discussion

Surface sterilization of explant is an important and most crucial step in starting plant tissue culture as it helps create a controlled and sterile environment for the growth of healthy plant tissues. By effectively eliminating surface contaminants, researchers can ensure the success of their experiments and maintain the integrity of their cultures.

Unoptimized concentrations of the sterilizing agent can have a lethal or harmful effect on the explant viability and can restrict the growth and development of the explant. Therefore, suitable concentration, combinations, and duration of exposure of sterilant are essential to raising *in vitro* cultures successfully and effectively.

In the present study, various sterilizing agents ($HgCl_2$, EtOH, NaOCl, and $AgNO_3$) were tested at different concentrations and durations of surface contact for *A. indica* leaves. The results indicated that the treatment with 0.1% $HgCl_2$ for 5 minutes was the most effective among the tested agents. This treatment yielded a survival rate of 90% for the explants, with only 10% experiencing contamination-related death. These findings are consistent with previous studies that have reported the efficacy of 0.1% $HgCl_2$ as a sterilant for *A. indica*, as well as the use of streptomycin (0.1%) and bavistin (0.1%) (Rathore *et al*., 1994; Arya *et al*., 1995; Venkateswarlu *et al*., 1998; Arya and Arya, 1998; Chaturvedi *et al*., 2004; Quraishi *et al*., 2004; Arora *et al*., 2010). In contrast, NaOCl at 5% for 15 minutes and EtOH at 70% for 3 minutes showed moderate effectiveness but with higher death rates of explants, indicating potential

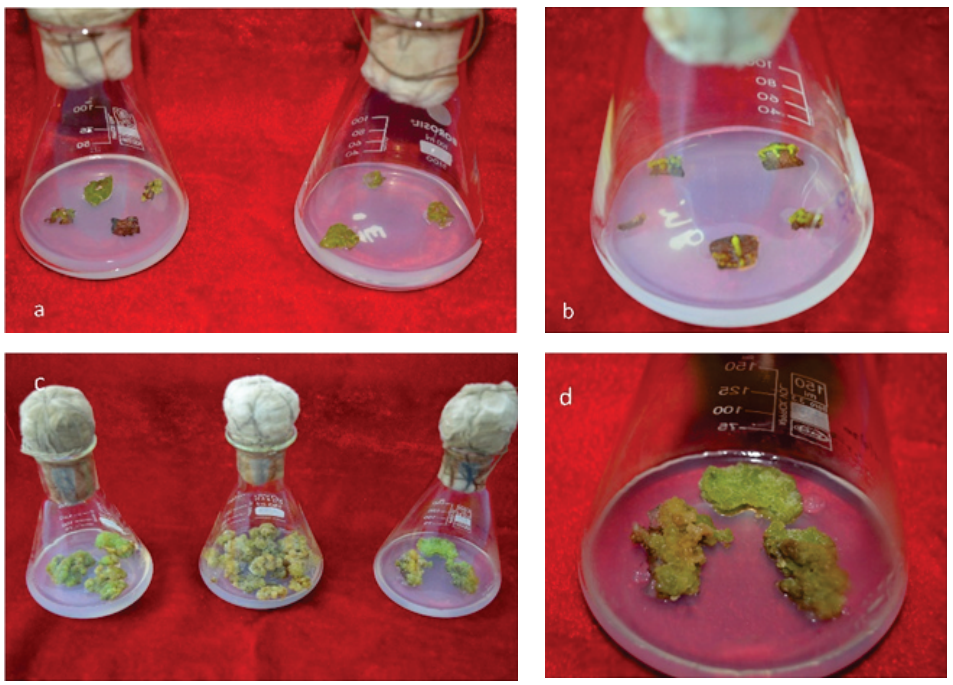

Fig. 7. (a) and (b) Depiction of few explants with minimal or no contamination, (c) and (d) Explants with noticeable growth, possibly indicating successful culture or proliferation

toxicity. AgNO3, particularly at 2% for 10 minutes, resulted in a fair survival rate but with noticeable contamination and toxicity. These findings underscore the necessity of optimizing sterilization conditions to achieve a balance between efficacy and explant health, particularly in the context of neem species, where such studies are not extensively documented. The results highlight $HgCl_2$ as a preferable sterilizing agent under the tested conditions, offering valuable insights for future research in plant tissue culture and sterilization methodologies.

Conclusion

The comparative analysis of four sterilizing agents, as illustrated in Fig. 1 and Table 2, clearly indicates that $HgCl_2$ at a 0.10% concentration for 5 minutes is the most effective in promoting explant survival with minimal contamination-related death in *Azadirachta indica*. This finding underscores the superiority of $HgCl_2$ in balancing sterilization efficacy and explant health. In contrast, $AgNO_3$ at a 2% concentration for 10 minutes, while yielding a decent survival rate, was associated with the highest rate of contamination-related deaths among the tested sterilants. The present study goes beyond simply reaffirming the efficiency of 0.1% $HgCl_2$ as a sterilant for *A. indica*, by conducting a comprehensive comparison of several sterilants including EtOH, NaOCl, and $AgNO_3$ at different concentrations and contact times. While the effectiveness of 0.1% $HgCl_2$ is already recognized in existing literature, our research provides a more detailed evaluation of the relative efficacy of various sterilizing agents under diverse conditions. This expanded insight is crucial in guiding future experimental designs and offering alternative solutions where the use of $HgCl_2$ might be constrained or unsuitable.

Acknowledgements

Authors are thankful to the Director ICFRE-AFRI for providing facility to carry out this study and CAMPA Authority, MoEFCC, New Delhi for financial support.

Conflict of Interests

The Authors declare no conflict of interests.

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