Indian Journal of Plant Sciences ISSN: 2319–3824
Online, International Journal, Available at http://www.cibtech.org/jps.htm
2023 Vol.12, pp.106-113/Rehman et al.
Research Article (Open Access)

OPTIMIZATION OF *IN VITRO* STERILIZATION PROTOCOL OF ENDANGERED PLANT *ADANSONIA DIGITATA* L.

[1]M. Ur Rehman, [1]M. Chaudhary, [2]A. Arya and [1]S. Kumar*
[1]Department of Biotechnology, Shobhit Institute of Engineering and Technology (Deemed to-be-University) NH-58, Modipurum, Meerut- 250110, U.P., India
[2]Department of Biotechnology, Noida Institute of Engineering Technology, 19, Knowledge Park- II, Institutional Area, Greater Noida (UP), India
**Author for Correspondence: dr.sandeepkumar@shobhituniversity.ac.in*

ABSTRACT
Adansonia digitata L., a critically endangered species in India, requires tissue culture techniques for regeneration and preservation of genetic resources. To achieve healthy and viable explants in tissue cultures, it is essential to perform effective surface sterilization. Various sterilizing agents can be used to eliminate surface contaminants. The aim of this study was to identify the most effective disinfection method for shoot regeneration in *A. digitata* using shoot tips and nodal segments. Four different sterilization approaches were employed using different concentrations of $HgCl_2$ (0.1-2.0%), $Ca(OCl)_2$ (0.9-10%), NaOCl (0.5-5.0%), and H_2O_2 (3-5%), with varying times of 3, 5, 7, and 10 minutes, followed by four rinses with sterile distilled water. The nodal segment and shoot tip explants were then cultured on Murashige and Skoog (MS) basal medium with various permutations of auxin and cytokinin. Results revealed that $Ca(OCl)_2$ demonstrated strong bactericidal activity, but did not exhibit a strong potential as a sterilizing agent for nodal and shoot tips explants, along with NaOCl and H_2O_2. In contrast, $HgCl_2$ showed the best reduction in contamination and gave a significant result. Surface sterilization with 0.1% mercuric chloride for 5 minutes was identified as the optimum concentration and duration, resulting in the highest percentage of nodal segments and shoot tip explants survival and viability. Therefore, it can be concluded that mercuric chloride is the most effective surface sterilization agent for *Adansonia digitata* L. All viable explants successfully developed into shoots.

Keywords: *Adansonia digitata L., Mercuric Chloride, Endangered Plant, Surface Sterilization, Contamination*

INTRODUCTION
The African baobab tree, scientifically known as *Adansonia digitata* L., belongs to the Malvaceae family and Bombacoideae subfamily. The common name "monkey bread tree" is derived from the fact that monkeys eat its fruit. Baobabs are easily recognizable because of their unique and bizarre shapes and are the most prominent tree species in the hot and dry regions of tropical African countries. *Adansonia digitata* L. is an important tree species in Africa, with high nutritional and medicinal value.
Furthermore, due to its distinct phytochemical composition and the increased demand from the food industry, the commercial value of baobab has increased significantly during the past decade. Among many plant species used in ethno-medicinal practices, baobab is widely used in traditional medicine. The fruit part of *Adansonia digitata* L. is traditionally used to treat fever, dysentery, hemoptysis, diarrhea, and smallpox in humans (Donatien Kaboré, 2011; Mathaba, 2016). Additionally, leave infusion is used to treat diarrhea, kidney, and bladder diseases (Oloyede *et al*., 2013).
Natural propagation is one of the key methods for the propagation of baobab trees. The natural propagation of baobab occurs through seed germination and vegetative propagation.
The seeds are contained in hard-shelled pods, which fall from the tree and can remain viable for several years. The germination of seeds can be influenced by factors such as seed coat thickness and
the presence of dormancy mechanisms.

Vegetative propagation is also an important method for the propagation of baobab trees. It occurs through the formation of adventitious roots from cuttings, layering, and suckering. The success of vegetative propagation can be influenced by factors such as the age of the parent tree, the season of collection, and the method of propagation. Proper management practices, such as appropriate selection of seed sources and vegetative propagation methods, can enhance the success of natural propagation of baobab trees (N'Doye *et al*., 2012).

To meet growing demands, it is imperative that plants be produced on a big scale, making tissue culture techniques a more reliable source. The key benefits of *In vitro* propagation techniques over traditional propagation methods are the ability to multiply rapidly under controlled conditions for a wide variety of species (Smith and Drew, 1990). The use of micropropagation has been studied in African baobab tree (*Adansonia digitata* L.) with promising results (Rolli *et al*., 2016). *In vitro* propagation of baobab has been reported through the culture of different explants, such as leaves, petioles, hypocotyls, cotyledonary nodes, and nodal segments, on various media formulations supplemented with plant growth regulators (PGRs) (Gebauer *et al*., 2016).

Research has been conducted to optimize the protocol for efficient micropropagation of baobab. For example, one study showed that 1-naphthaleneacetic acid (NAA) and benzyl aminopurine (BAP) were the most effective PGRs for inducing shoot proliferation in shoot-tip explants of baobab (Ishii and Kambou, 2007). Other studies have reported successful micropropagation using other PGR combinations, such as indole-3-acetic acid (IAA) and kinetin (KN) (Rolli *et al*., 2016). In addition, the use of various types of explants and culture conditions have also been explored to optimize the micropropagation protocol for baobab.

Sterilization of explants is an essential step in *In vitro* propagation of the African baobab tree (*Adansonia digitata* L.) as it helps in eliminating surface contaminants and prevents fungal and bacterial infections. Various sterilization methods have been employed for baobab explants, including the use of sodium hypochlorite, mercuric chloride, and hydrogen peroxide at varying concentrations and durations.

Different methods of sterilization have been reported for different explants, such as shoot tips, internodal segments and petioles. One method that has been optimized for two-node segments of baobab is to rinse them with fresh water and Tween-20 followed by washing with a solution containing Tween 20 (0.025% v/v), Bavistin (1% w/v) and Tetracycline (0.004% w/v) for 1 h. This method reduces callus formation and increases the formation of microshoots when combined with cytokinin and indole-3-butyric acid.

The aim of the current work was to develop a methodology for the surface sterilization of *Adansonia digitata* L. shoot tip and nodal segment explants using a variety of sterilizants.

MATERIALS AND METHODS

It is essential to sterilize explants thoroughly to avoid contamination and ensure the success of *In vitro* propagation. As it is crucial to determine the appropriate combination of sterilizing agents and concentrations to achieve optimal results. In order to optimize the method of sterilization different sterilizing agents invarious concentrations were used to test the efficiency of sterilization and subsequent shootlet development.

Source and Collection of plant materials

Healthy and young shoot (Approx 2 to 4 cm long), containing axillary buds (Second, third, fourth nodes: from shoot apex) were excised and collected from 6 years old tree grown in Shobhit Deemed University, Meerut by cutting with sterile scissor and collected in a round conical flask.

Explant Surface Sterilization

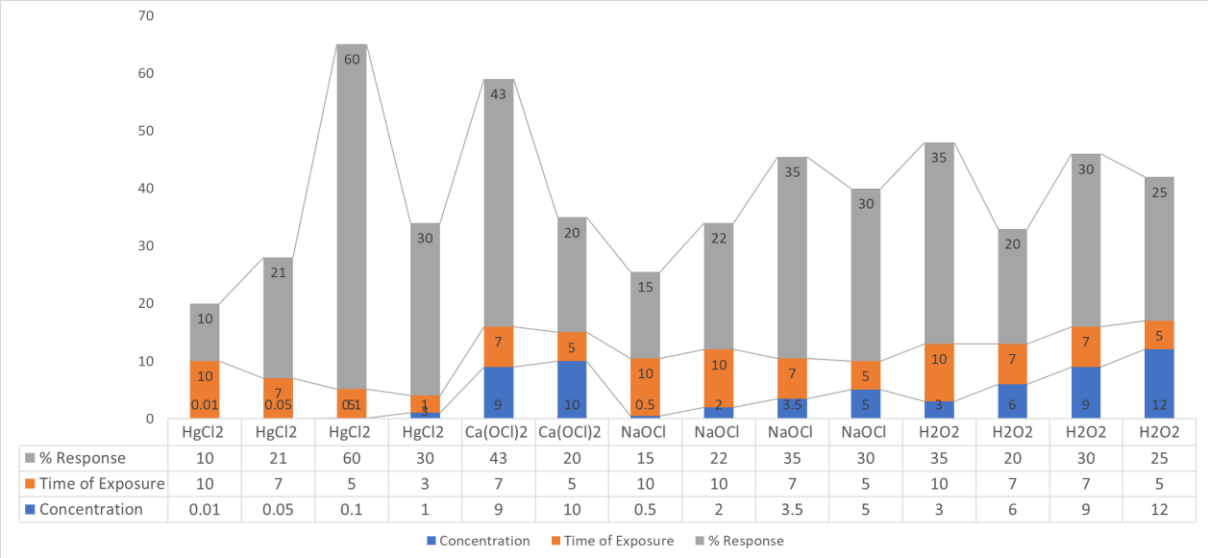
The process of sterilizing explants for *In vitro* propagation of *Adansonia digitata* L. involves several steps. First, freshly harvested plant material with 2-3 nodal segments and shoot tips is washed for 10-15 minutes under a continuous stream of running tap water to remove any dirt or debris. Then, the explants are washed again with liquid detergent (Tween 20) to remove any remaining surface contaminants. The next step involves treating the explants with 70% ethyl alcohol for 30 seconds and then drying them in a laminar flow.

After this, the explants are incubated in different concentrations and durations of sterilizing agents such as $HgCl_2$, $Ca(OCl)_2$, NaOCl, and H_2O_2. The concentrations of these agents range from 0.1-2.0%, 0.9-10%, 0.5-5.0%, and 3-5%, respectively, and the incubation times vary from 3 to 10 minutes. The explants are then washed with sterilized distilled water four times to remove any traces of the sterilizing agents.
After sterilization, the explants were inoculated on MS medium supplemented with different concentrations of BAP and NAA to induce shoot development. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 15 minutes at 121°C and 15 lbs pressure. The *In vitro* cultures were maintained under controlled growth room conditions at 25±2°C and 60-70% relative humidity with a light intensity of 3000 lux and a photoperiod of 16 hours of daylight and 8 hours of darkness. Each experiment was conducted at least thrice to ensure consistency and reliability of results.
Contamination percentage and survival rates were recorded to determine the effectiveness of the sterilization process.
Contamination percentage was calculated using the formula,

$$\text{Contamination percentage (\%)} = \frac{Number\ of\ contamiated\ cultures}{Total\ number\ of\ incubated\ cultures}$$

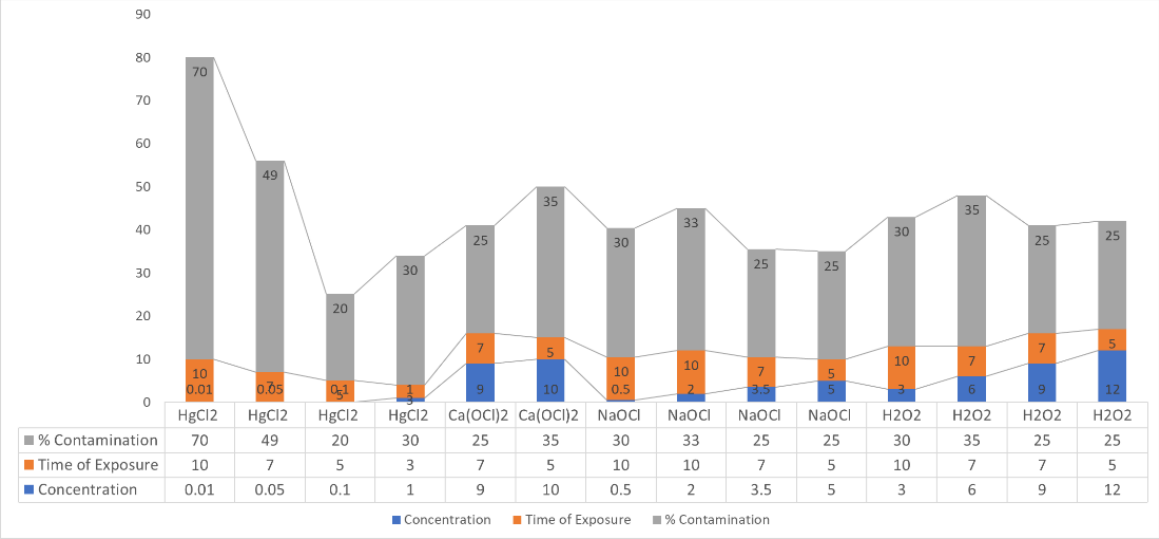
Statistical analysis
Mean % Response: calculated as the average of all % Response values for each sterilization agent. Mean % Contamination: calculated as the average of all % Contamination values for each sterilization agent. Mean Most Effective Time: calculated as the average of all Most Effective Time values for each sterilization agent. Standard Deviation: calculated as a measure of the amount of variation or dispersion in the % Response, % Contamination, and Most Effective Time values for each sterilization agent.

RESULTS AND DISCUSSION
Before inoculating the explant tissues on the nutritional medium, it is crucial to perform an optimal surface sterilization to remove any microbial contaminants present on the plant surfaces. By removing surface contaminants like dust and grime, the initial application of tween 20, a wetting agent and surfactant, promotes disinfection



Graph 1:Effect of different sterilizing agents time of treatment on the bud induction response from the nodal segments of *Adansonia digitata*.

The graphs below demonstrate the results of using various sterilizing agents. The Graph 1 and 2 provides information on the effect of different sterilizing agents on nodal segment explants of *Adansonia digitata*. The agents tested were $HgCl_2$, $Ca(OCl)_2$, NaOCl, and H_2O_2, and the parameters measured were the concentration (%), time of treatment (minutes), response (%), and contamination (%).



Graph 2: Effect of different sterilizing agents time of treatment on the contamination percentage from the nodal segments of *Adansonia digitat*.

The results also show that the concentration and time of treatment of the sterilizing agents can have a significant effect on the response rate and contamination of plant explants. For example, $HgCl_2$ at a concentration of 0.1% for 5 minutes resulted in a 60% response rate and 20% contamination, whereas at a concentration of 1.0% for 3 minutes, it resulted in a lower response rate of 30% but a higher contamination rate of 30%. This is consistent with previous research that has shown that the concentration and time of treatment of sterilizing agents can affect the viability and growth of plant explants (Mihaljevic *et al*., 2013; Thompson *et al*., 2009; Tiwari *et al*., 2012).

$Ca(OCl)_2$was also found to be effective in sterilizing nodal segments, with a response rate of 43% at a concentration of 9% and a treatment time of 7 minutes. However, the contamination rate was relatively high at 25%. At a concentration of 10%, $Ca(OCl)_2$ showed a lower response rate of 20% and a higher contamination rate of 35%. This suggests that a concentration of 9% may be optimal for sterilizing nodal segment explants of *A. digitata*.

NaOCl showed moderate effectiveness, with a response rate ranging from 15% to 35% depending on the concentration and treatment time. The contamination rate was also moderate, ranging from 25% to 33%. The optimal concentration for NaOCl was found to be 3.5%, with a treatment time of 7 minutes. This result is also consistent with previous studies that have shown that NaOCl is not as effective as other sterilizing agents for plant explants (Mihaljevic *et al*., 2013; Thompson *et al*., 2009).

H_2O_2 showed relatively high effectiveness, with response rates ranging from 20% to 35% and contamination rates ranging from 25% to 35%. The optimal concentration for H_2O_2 was found to be 3%, with a treatment time of 10 minutes.

The findings suggest that $HgCl_2$ at a concentration of 0.01% for 10 minutes is the most effective sterilizing agent for this plant species (Figure 1). However, the results also indicate that the concentration and time of treatment of the sterilizing agents should be carefully considered to achieve the best results.

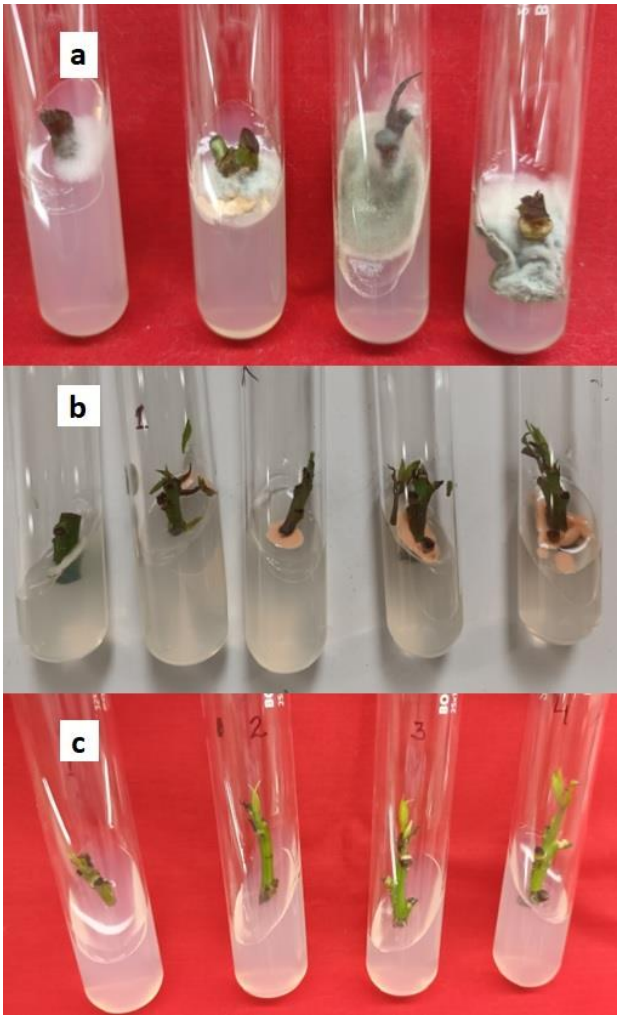
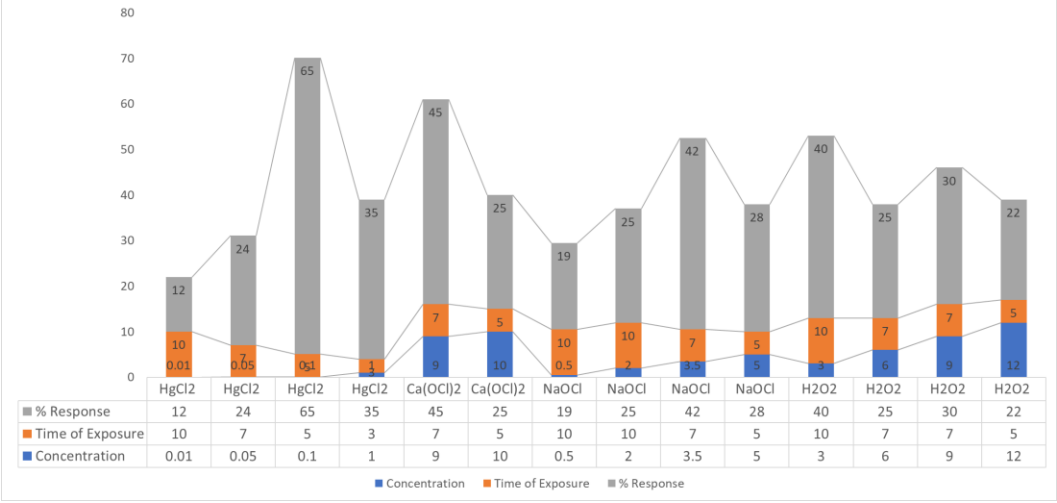


Figure 1: *(*a) Fungal contamination and (b) bacterial contamination in the nodal segments of *Adansonia digitata L.*; contamination free cultures after the treatment of $HgCl_2$.

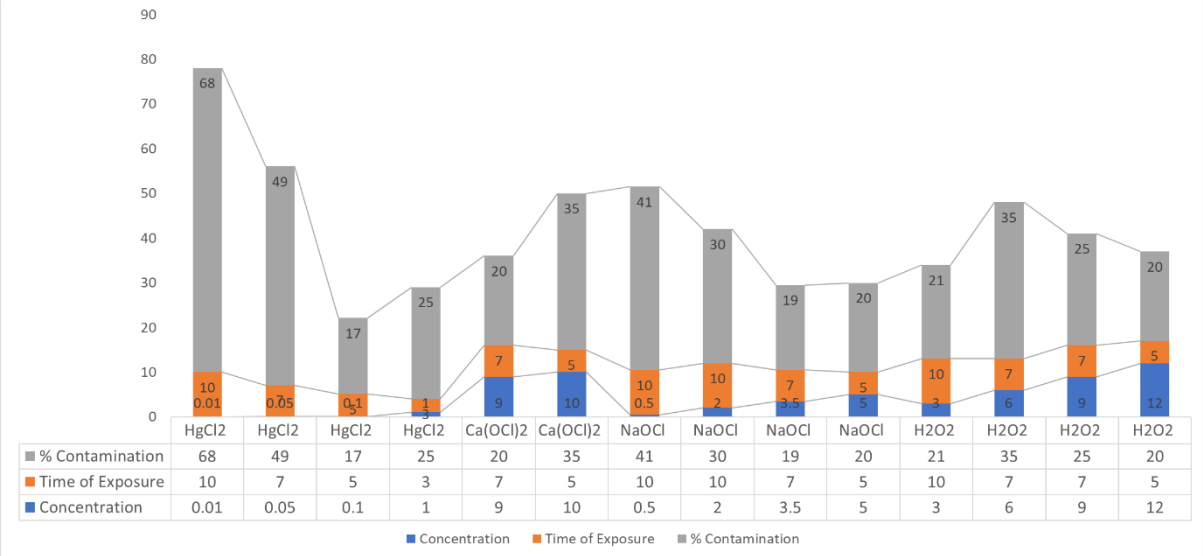


Graph 3: Effect of different sterilizing agents time of treatment on the contamination percentage from the shoot tip explant of *A. digitata*.

Graphs 3 and 4 display the response of different sterilization agents on shoot tips explants of *A. digitata*. The efficacy of the sterilization agents varied with concentration, time of treatment, and type of agent. Among the tested agents, $HgCl_2$ and $Ca(OCl)_2$show higher effectiveness in reducing contamination at their respective optimum treatment times of 5 and 7 minutes.

At lower concentrations and longer treatment times, NaOCl and H_2O_2 show comparable efficacy to $HgCl_2$ and $Ca(OCl)_2$, respectively. These findings suggest that the choice of sterilization agent should be based on the type of explant, the level of contamination, and the treatment time and concentration required for effective sterilization.

Previous research has also reported the efficacy of various sterilization agents for different explants. Previous investigations also demonstrated that $HgCl_2$ and NaOCl alone or in combination are effective at decreasing contamination (Gunawardhana *et al*., 2022; Urbi and Zainuddin, 2015; Wolella, 2017).



Graph 4: Effect of different sterilizing agents time of treatment on the bud induction response from the shoot tip explant of *A. digitata*.

$HgCl_2$ and $Ca(OCl)_2$at their respective most effective treatment times showed the highest percentage of contamination-free explants. The concentration of 0.01% $HgCl_2$ for 10 minutes treatment time resulted in 68% contamination-free explants, while 9% $Ca(OCl)_2$for 7 minutes treatment time resulted in 45% contamination-free explants.

NaOCl and H_2O_2 showed comparable effectiveness to $HgCl_2$ and $Ca(OCl)_2$ at lower concentrations and longer treatment times. For NaOCl, 2% concentration for 10 minutes treatment time resulted in 25% contamination-free explants, while for fH_2O_2, 6% concentration for 7 minutes treatment time resulted in 35% contamination-free explants. In several other studies it has been reported that the use of NaOCl and $H_2O_2$are the most effective in reducing contamination (Lineback *et al*., 2018). These variations in the effectiveness of sterilization agents may be attributed to differences in the type of explant, level of contamination, and environmental conditions.

CONCLUSION

In conclusion, the choice of sterilization treatment for plant tissue culture should be based on the type of explant, the level of contamination, and the duration of exposure required for effective sterilization. It is also important to consider the potential negative effects of the sterilization agents on plant growth and development. Further research is needed to optimize sterilization protocols for different plant species and tissue types.

Based on the results of the study, it can be concluded that the most effective surface sterilization agent for *Adansonia digitata* L. is 0.1% mercuric chloride for 5 minutes. This sterilization method resulted in the highest percentage of survival and viability for nodal segments and shoot tip explants. The other sterilizing agents, including $Ca(OCl)_2$, NaOCl, and H_2O_2, did not exhibit strong potential as effective sterilizing agents for nodal and shoot tips explants. Therefore, it is recommended to use mercuric chloride for the surface sterilization of A. digitata in tissue culture techniques for regeneration and preservation of genetic resources. The successful development of all viable explants into shoots indicates the effectiveness of the identified sterilization method in achieving healthy and viable explants in tissue cultures.

ACKNOWLEDGMENTS
The authors would like to thank the Department of Biotechnology, Shobhit Institute of Engineering and Technology (Deemed to-be-University) NH-58, Modipurum, Meerut -250110, U.P., India for their valuable support.

REFERENCES
DonatienKaboré (2011). A review of baobab (*Adansonia digitata*) products: Effect of processing techniques, medicinal properties and uses. *African Journal of Food Science*, **5**(16) 833–844.
Gebauer J, Adam YO, Sanchez AC, Darr D, Eltahir MES, Fadl KEM, Fernsebner G, Frei M, Habte TY, Hammer K, Hunsche M, Johnson H, Kordofani M, Krawinkel M, Kugler F, Luedeling E, Mahmoud TE, Maina A, Mithöfer D, Munthali CRY, Noga G, North R, Owino WO, Prinz K, Rimberia FK, Saied A, Schüring M, Sennhenn A, Späth MA, Taha MEN, Triebel A, Wichern F, Wiehle M, Wrage-Mönnig N and Kehlenbeck K (2016). Africa's wooden elephant: the baobab tree (*Adansonia digitata* L.) in Sudan and Kenya: a review. *Genetic Resources and Crop Evolution*, **63**(3) 377–399.
Gunawardhana WPDS, Perera PIP, Muhandiram HMAP, Swarnathilaka DBR and Priyadarshani KDN (2022). Surface sterilization protocols of leaf and bud explants for initiating *in vitro* cultures of *Piper nigrum* L. (Pepper). *Tropical Agriculture Research and Extension*, **25**(2) 132.
Ishii K and Kambou S (2007). *In vitro* culture of an African multipurpose tree species: *Adansonia digitata* L. *Propagation of Ornamental Plants*, **7**(2) 62–67.
Lineback CB, Nkemngong CA, Wu ST, Li X, Teska PJ and Oliver HF (2018). Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against Staphylococcus aureus and Pseudomonas aeruginosa biofilms than quaternary ammonium compounds. *Antimicrobial Resistance and Infection Control*, **7**(1).
Mathaba MG (2016). Ethnobotanical study of the cultural value and preservation status of *Adansonia digitata* (baobab species) among Vhavenda of Sagole Community in the Limpopo Province. *Doctoral dissertation, University of Limpopo.*
Mihaljevic I, Dugalic K, Tomas V, Viljevac M, Pranjic A, Cmelik Z, Puskar B and Jurkovic Z (2013). *In vitro* sterilization procedures for micropropagation of 'oblacinska' sour cherry. *Journal of Agriculture Science and Belgrade*, **58**(2) 117–126.
N'Doye AL, Sambe MAN and Sy MO (2012). Propagation of *African baobab* (*Adansonia digitata* L., Bombacoideae, Malvaceae) germplasm through *in vitro* cloning. *Advanced Environmental Biology*, **6**(10) 2749–2757.
Oloyede GK, Adaramoye OA and Oguntokun OJ (2013). Phytochemical and hepatotoxicity studies on *Adansonia digitata* leaf extracts. *Journal of Experimental and Applied Animal Sciences*, **1**(1) 25.
Rolli E, Brunoni F and Bruni R (2016). An optimized method for *in vitro* propagation of African baobab (*Adansonia digitata* L.) using two-node segments. *Plant Biosystematics*, **150**(4) 750–756.
Smith MK and Drew RA (1990). Current Applications of Tissue Culture in Plant Propagation and Improvement. *Functional Plant Biology*, **17**(3) 267–289.

Thompson IM, Laing MD, Beck-Pay SL and Fossey A (2009). Screening of topical sterilants for shoot apex culture of *Acacia mearnsii*. *South African Forestry Journal*, **71**(1) 37–40.
Tiwari S, Arya A and Kumar S (2012). Standardizing sterilization protocol and establishment of callus culture of sugarcane for enhanced plant regeneration *in vitro*. *Botanical Research Journal*, **7**(1) 1-7.
Urbi Z and Zainuddin Z (2015). Standardization of surface sterilization protocol of field grown stevia rebaudiana prior to *in vitro* clonal propagation. *Journal of Teknologi*, **77**(24) 141–146.
Wolella EK (2017). Surface sterilization and *in vitro* propagation of *Prunus domestica* L. cv. Stanley using axillary buds as explants. *Journal of Biotech Research*, **8**(1) 18–26.