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In vitro Propagation of *Lagenaria Siceraria*: A Plant of the Cucurbitaceae Family with High Medicinal Value

Theophilus Kabiru [a] and Peter K. Njenga [a*]

[a] Jomo Kenyatta University of Agriculture and Technology, School of Biological Sciences, P.O. Box 62000-00200, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background and Objective: *Lagenaria siceraria* is extensively grown as a vegetable crop in tropical and subtropical regions of the world as a source of food and for it's medicinal potential. The purpose of this research was to determine the optimal conditions for plantlet regeneration of *Lagenaria siceraria* microshoots on various concentrations of benzyl amino purine and naphthalene acetic acid *in vitro*.
Materials and Methods: The research was designed as a completely randomized design. Shoot tips of *Lagenaria siceraria* were surface sterilized with sodium hypochlorite(NaOCl) at different concentrations (0,3,4,5,6 and 7 mg/L) and cultured on Murashige and Skoog (MS) solidified medium supplemented with 6-benzylaminopurine (BAP) at different concentrations of (0,0.5,1.0,1.5,2.0,2.5 and 3.0mg/L) for shoot induction and the obtained shoots were transferred to MS medium containing naphthaleneacetic Acid(NAA) at different concentrations (0,0.5,1.0,1.5,2.0,2.5 AND 3.0mg/L) for root induction.

**Corresponding author: E-mail: pknjenga@jkuat.ac.ke, pknjenga22@jkuat.ac.ke;*

Results: Surface disinfection was optimal after immersion of shoot tips and/or nodal explants in cleaning detergent (5 min), ethanol 70% (1 min), and sodium hypochlorite 5mg/L (15 min). MS medium supplemented with 2.0 mg/L BAP was most effective in inducing shoot multiplication (100%), with an average of 4,33 shoots per explant after 4 weeks. MS medium supplemented with 2.0 mg/L NAA was most appropriate for rooting (100%) with an average of 5.33 roots per shoot after 4 weeks.
Conclusion: shoots sterilized with 5mg/L NaOCl and inoculated in MS medium supplemented with 2.0 mg/LBAP and 2.0 mg/L NAA were optimal conditions for the surface sterilization, shoot and root induction of *Lagenaria siceraria* shoots.

Keywords: Micropropagation; MS medium; In vitro; surface sterilization; shooting; rooting.

1. INTRODUCTION

"*Lagenaria siceraria* also known as bottle gourd is a monoecious vine with long, ribbed stem, strong tendrils belonging to the Cucurbitaceae family. It is extensively cultivated in tropical and sub-tropical regions, notably for its medicinal value and source of food. The fruits of Bottle gourd have a variety of shapes: they can be huge and rounded, small and bottle-shaped, or slim and serpentine, and they can grow to be over a meter long. Rounder varieties are typically called calabash gourds" [1]. *Lagenaria siceraria*, is utilized in a separate system of traditional medicine to cure numerous ailments such as asthma, fever, malaria, hypertension, jaundice, ulcer, cardiac, bronchial and skin problems" [1]. "Cucurbitacin, a secondary metabolite found in the seeds and fruit sections of several cucurbits, has been described to have purgative, emetic, and anthelmintic actions. This category of chemicals had been considered for its anti-inflammatory, hepatoprotective, cytotoxic, and cardiovascular properties" [2]. The plant's fruiting body is well-liked for its taste and extraordinary nutritional content, which includes practically all of the needed ingredients for good health. The plant might provide physiologically active polysaccharides" [3]. The fruit is extensively used as a medicinal vegetable in Asia and Africa for a variety of ailments. Alternative medicine is made from several components of this plant, including the fruit, seed, leaf, and root" [4]. Traditional uses of the fruit include cardioprotective, antidote, aphrodisiac, cardiotonic, diuretic, and general tonic properties. The fruit juice is used as a cure for jaundice and it heals other liver ailments as it is a good source of anti-oxidants" [5]. *Lagenaria siceraria* is traditionally propagated via seeds. Propagation of *Lagenaria siceraria* via this method appears to be restricted due to its high susceptibility to fungal and bacterial diseases, labor intensiveness and time consuming with low survivability and possibility of growth and yield retardation. Furthermore, the recalcitrant nature of the seeds also causes difficulties in generating the plantlets from seed and the seed raised plants exhibit genetic variations" [6]. Hence, i*n vitro* propagation of bottle gourd serves as an alternative method in increasing productivity and developing a sustainable agricultural system. The pharmaceutical production could also be upregulated using plant tissue cultures under optimized conditions. These automated controls of tissue growth would definitely contribute to cost reduction and productivity improvement of plantlets with desirable traits. Moreover, current synthetic treatments have many adverse effects and are regarded as unsafe and disadvantageous in the treatment of human ailments" [7]. There is also an emergent concern about herbal medicines across the world, which is complemented by more laboratory research into the pharmacological characteristics of bioactive substances and their capacity to cure various disorders. Through ethnopharmacology and traditional medicine, a slew of new medications has made their way onto the worldwide market" [8]. Herbal medications are a viable alternative to current synthetic treatments owing to their few adverse effects and are regarded as safe and useful in the treatment of human ailments. The adaptability of *L. siceraria* to various ecological zones, its wide range of utilizations, and its high phenotypic plasticity make it a likely species to sustain a minimal yield in conditions of variable climate. Various studies to obtain a better protocol for bottle gourd propagation have been reported, such as sterilization techniques, shoot induction and rooting plant regeneration through organogenesis [9]. In a related study, pumpkin (*Cucurbita maxima*) and ash gourd (*Benincasa hispida*) explants have been effectively sterilized using 1% savlon (v/v) and two drops of tween-80 for 5 minutes treated with; 0.03% mercuric chloride (w/v) at 3.0 minutes for pumpkin and ash

gourd explants with 0.03% mercuric chloride(w/v) at 3.5 minutes. 80% of the explants were free from contamination and tissue killing [10]. Pumpkin and ash gourd reported optimum multiple shoot proliferation at a concentration of 2.0 mg/l BAP in pumpkin but in ash gourd it was 1.5 mg/l BAP. Therefore, this study aimed to determine the optimum sodium hypochlorite (NaOCl), 6-benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) for sterilization, shoot and root regeneration respectively in *Lagenaria siceraria*.

2. MATERIALS AND METHODS

Study area: This experiment was conducted at the Botany Laboratory, Jomo Kenyatta University of Agriculture and Technology.

Research tools: The tools used in this research are autoclave, distilled water, analytical scale, pH meter, Laminar Air Flow Cabinet (LAFC), culture bottle, measuring beaker, tweezers, scalpel, surgical blade, hand sprayer, pipette, stirring rod, culture shelf, Bunsen burner, 60 mesh filter and documentation tool. Planting materials used are seeds of *Lagenaria siceraria*. Other materials used include MS medium, NAA, BAP, sterile distilled water, sucrose, phytagel, 70% alcohol, detergent, tween 20 and Bayclin (bleach contains 5.25 % NaOCl).

Research protocol: This study was prepared based on a Complete Randomized Design (CRD) with two factors. The first factor was the concentration of sodium hypochlorite solution consisting of six levels i.e., without sodium hypochlorite (NaOCl) 0 (T0), 3Mg/l NaOCl (T1), 4Mg/L NaOCl(T2),5 Mg/L NaOCl(T3), 6 Mg/L NaOCl(T4) and 7Mg/L NaOCl(T5). The second factor is the concentration of BAP, which consists seven levels i.e., without BAP (B0), 0.5 ppm BAP (B1), 1.0 ppm BAP (B2), 1.5 ppm BAP (B3),2.0 ppm BAP (B4), 2.5 ppm BAP (B5) and 3.0 ppm BAP (B6). The third factor is concentration of NAA which consists of seven levels i.e., without NAA (N0), 0.5 ppm of NAA(N1), 1.0 ppm of NAA(N2), 1.5 ppm NAA(N3), 2.0 ppm NAA(N4), 2.5 ppm NAA(N5), 3.0 ppm NAA(N6). Thus, there are twenty combinations of treatments. Each treatment combination was repeated three times, so there were 60 experimental units. Each experimental unit was composed of one explant, resulting in a total sample of 60 explants. The observation parameters included the average number of sterile explants, shoot numbers and root numbers. All these parameters were monitored at 7, 14,21 and 28 Days after culturing. The data obtained were analyzed using an analysis of variance (ANOVA) and F test at 5% was carried out to determine the effect of treatments on the observed parameters. If it was significant, then a 5% Honestly Significance Difference (HSD) test was used to separate the significantly different means.

Seed planting: The seeds were planted on a tray containing sand soil. Watering was done every two days until the shoots were harvested to provide explants for subsequent *in vitro* manipulations.

Explant sterilization: Shoot-tips (1–1.5cm) were detached with scalpels from the mother plants above to proceed with the disinfection as follows: They were placed in a beaker and washed with running tap water to remove any adhering contaminants until clean, then shaken in a detergent solution to which 1–2 drops of surfactant (Tween 20) was added and shaken vigorously for 15 minutes at 90rpm. The shoots were then rinsed in running water until free of foam, then rinsed with sterile distilled water several times. Sterilization was continued on the laminar airflow using 70% ethanol (CH_5OH) for 1 min. The ethanol was then decanted and the explants immersed into 0,3,4,5.6 and 7% sodium hypochlorite solution for 15 minutes under continuous agitation. The explants were then rinsed with sterile distilled water 3 times and placed individually in borosilicate glass test tubes containing Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30g/L sucrose and 6g/L plant agar. The medium pH value was adjusted to 5.7-5.8 prior to autoclaving at 121℃ for 15 min. shoots were transferred onto MS medium without any plant growth regulator for 2 weeks. The cultures were maintained in a growth chamber under 16h/8h light/dark photoperiod, temperature of 25 ± 1 ◦C (day/night), 80% RH, and cool white fluorescent light intensity. The percentage of contaminant-free explants were recorded after 14 days of culture.

***In vitro* shoot multiplication:** Single, 1.0 cm-long shoot tips were cultured on the MS medium supplemented with a concentration of BAP (0mg/L,0.5mg/L, 1.0mg/L, 1.5Mg/L, 2.0mg/L, 2.5mg/L and 3.0 mg/L). The numbers of shoots were counted after every 4 weeks.

Root induction: Single shoot tips 1.0 cm in length were cultured on the MS medium

supplemented with NAA at the following concentrations: 0mg/L, 0.5mg/L, 1.0mg/L, 1.5mg/L, 2.0mg/L, 2.5mg/L, and 3.0mg/L. The number of roots and their lengths were recorded after 4 weeks.

3. RESULTS AND DISCUSSION

3.1 Results

Surface sterilization of shoots explants: In the present study, the analysis of variance showed significant effects of surface sterilization treatments in reducing the incidence of microbial contamination in shoot tip explants of *Lagenaria siceraria* (p=.05). Surface sterilization using sodium hypochlorite at 5mg/L proved to be effective in reducing the incidence of microbial contamination in shoot tip explants. For treatment 0 – 4 mg/L, the survival percentage of cultured explants significantly increased as the concentration of sodium hypochlorite used for surface sterilization treatment increased. However, the detrimental effects of higher concentrations of sodium hypochlorite sterilization treatments was evident as the concentration was increased to 6 and 7 mg/L. At these higher concentrations, the explants got 100% bleaching. Therefore, surface sterilization with 70% (v/v) ethanol for 1 min followed by 5% mg/L sodium hypochlorite for 15 min was the preferred method for *Lagenaria siceraria* shoots, as it managed to have no contamination (0%) with low bleaching (0%) and high survivability (100%) after two weeks of surface sterilization (Table 1 and Fig. 2).

For treatment 0, the contamination percentage of shoot explants (100%) was the highest than the rest of the treatments. Microbial contamination was observed in the MS medium between 3 and 14 days after surface sterilization of explants. It was noted that both bacterial and fungal pathogens grew rapidly in this study and colonized the culture media, which resulted in increased tissue mortality.

Table 1. Effects of surface sterilization method and duration on the contamination, bleaching and survival of shoot explants, after 14 days of culture

Explant treatment	Contaminated percentage %	survived percentage %	Bleached percentage %
Control (T0)	100.00	0.00	0.00
3mg/L NaOCl	66.37	33.33	0.00
4mg/L NaOCl	33.33	66.37	0.00
5mg/L NaOCl	0.00	100.00	0.00
6mg/L NaOCl	0.00	0.00	100.00
7mg/L NaOCl	0.00	0.00	100.00

Values expressed as the mean of three replicated experiments, each indicating a significant difference (p=.05)

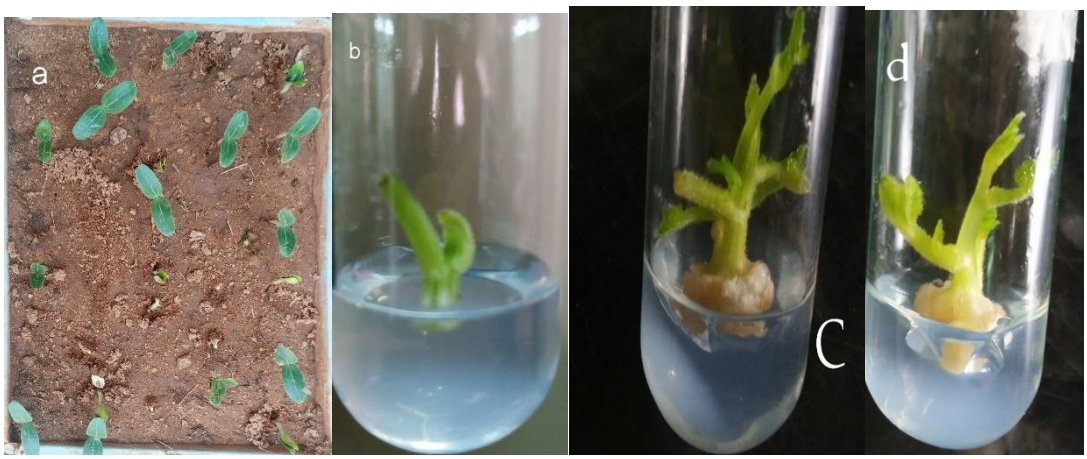

Fig. 1(a-d). *Lagenaria siceraria* seedlings and cultured shoots (a) *Lagenaria siceraria* seedlings actively growing in a tray of soil (b) control experiment for shooting after 28 days (c) shoots cultured in MS media supplemented with 1.5mg/L BAP after 28 days (d) shoots cultured on MS media supplemented with 2.0mg/L BAP after 28 days

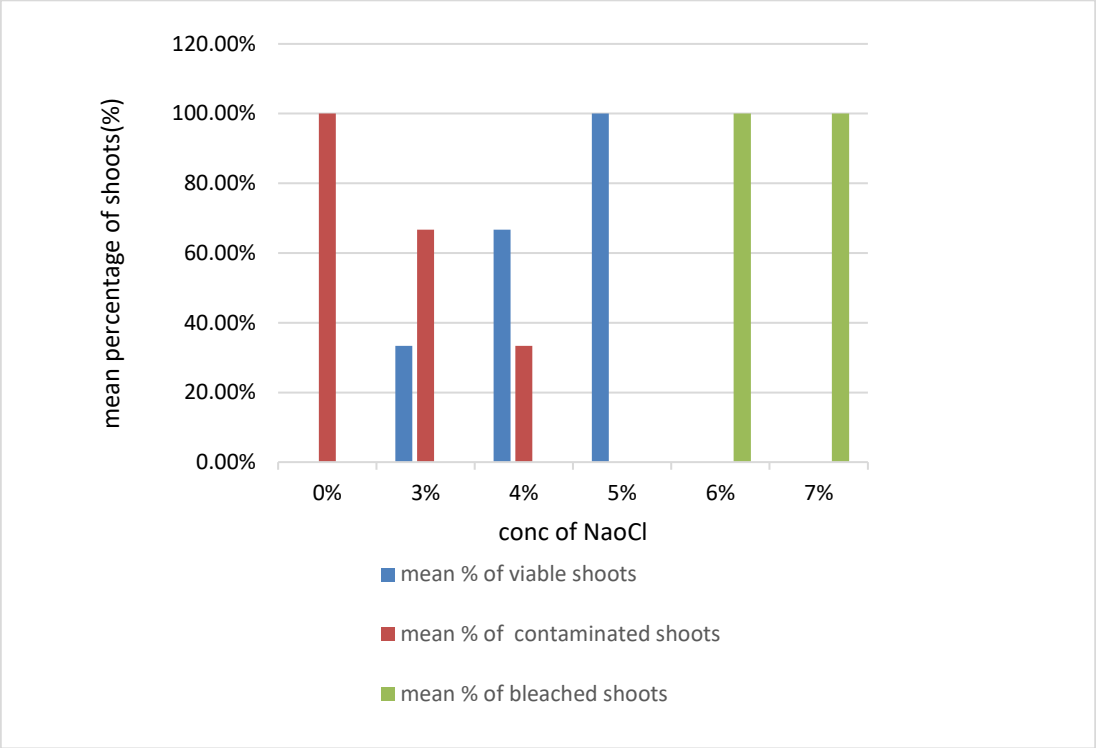

Fig. 2. Effect of different concentrations of sodium hypochlorite on sterilization of shoots

Shoot multiplication: Shoot multiplication was strongly influenced hormonal treatments (p=.05) (Table 2). All explants were found to be responsive towards the tested hormonal treatments with the exception of the control treatment, where there was no shooting response recorded (Table 2, Fig. 3 and Fig. 4). Of the different hormonal concentrations tested, the optimum percent response (100%) was observed on MS media supplemented with 2.0mg/L, BAP, with the average shoot numbers being 4.33±0.33 shoots/explant. Media with 0.0mg/L of BAP showed (0%) response and no multiple shoots were formed. In order to increase the shoot multiplication, BAP (0.5–3.0 mg/L) were tested. An increase in the number of shoots (0.67±0.33) was observed when the *in vitro* shoot explants were cultured on MS medium supplemented with BAP (0.5 mg/L). Increased concentration of BAP from 0.5mg/L up to 2.0 mg/L led to an increase in shoot number. 23.26%, 46.51%, and 70.00% response rate was observed in 0.5,1.0 and 1.5mg/l of BAP respectively with the average shoot number being 0.67±0.33,2.33±0.33 and 3.00±0.33 shoots per explant respectively. At higher concentrations of BAP beyond the optimum (2.5 and 3.0 mg/L), explants produced fewer number of adventitious shoots.

Table 2. Effect of plant growth regulator BAP added to MS media on in vitro shooting of Lagenaria siceraria

BAP concentration(mg/L)	Responding shoots %	Average shoot number/ explant
0	0.00	0.00±0.33
0.5	23.26	0.67±0.33
1.0	46.51	2.33±0.33
1.5	70.00	3.00±0.33
2.0	100.00	4.33±0.33
2.5	46.51	2.33±0.33
3.0	30.23	1.00±0.33

Data is represented as mean ± standard error of mean (SEM). Mean in each column is not significantly different at p = .05 by Duncan's MRT at 5% level of significance

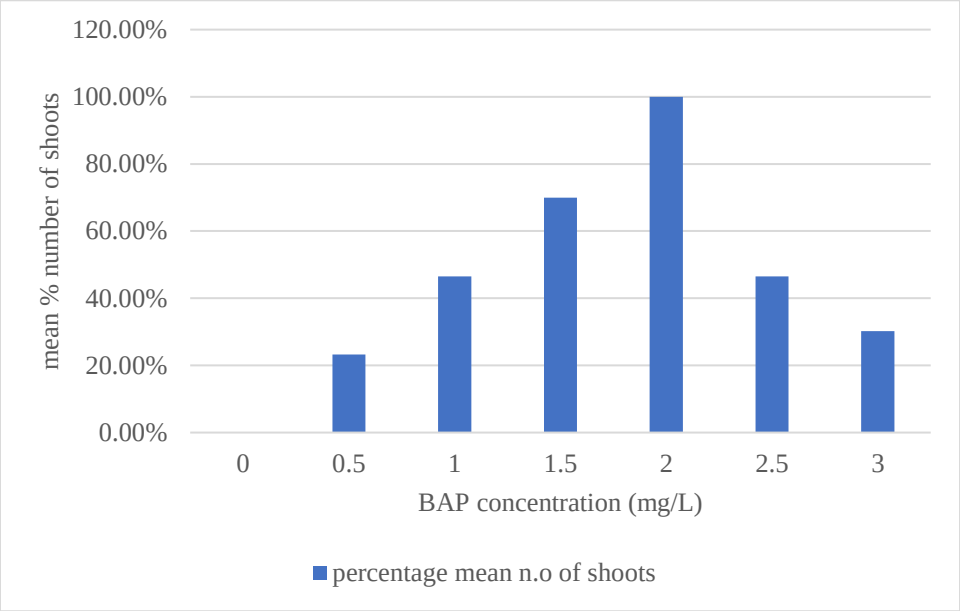

Fig. 3. Graph showing BAP concentration against mean percentage number of shoots

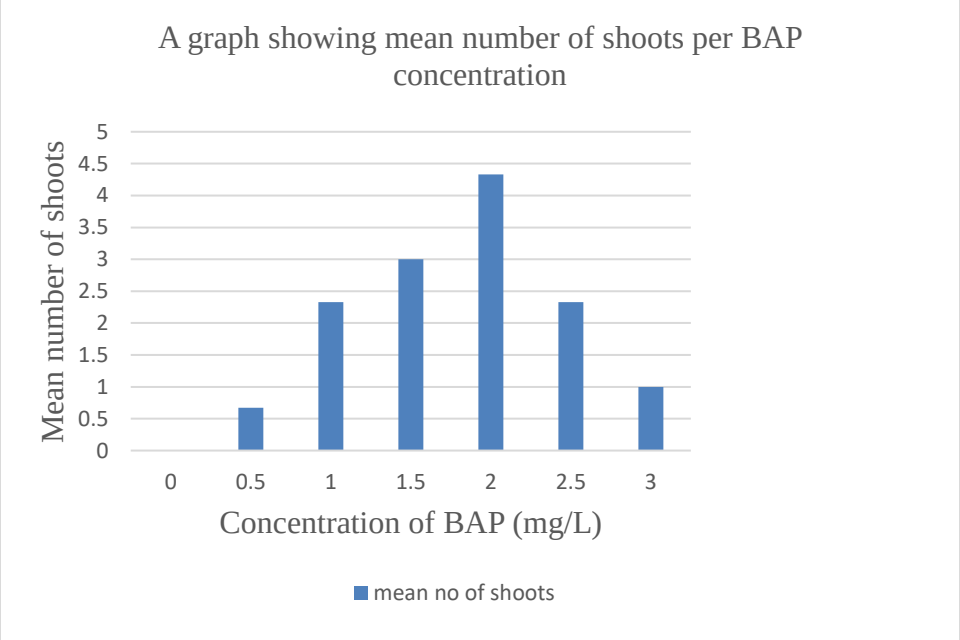

Fig. 4. Graph showing BAP concentration against mean number of shoots

Root induction: The number of rooted shoots significantly increased from 0.00-100.00%, as the concentration of NAA increased from 0.0-2.0 mg/L. *In vitro* shoot tips of *Lagenaria siceraria* were cultured on MS medium supplemented with NAA in the range 0.0–3.0mg/L. The number of roots obtained in the control treatment using hormone-free MS was significantly lower than those obtained from 0.5mg/L to 3.0 mg/L NAA. Among the various concentrations of NAA tested 2.0mg/L of NAA proved to be the most effective for root induction (5.33 ± 0.83 roots per explant). The media supplemented with 0.5 mg/L,1.0mg/L and 1.5 mg/L NAA promoted root production to 1.67, 2.67 and 3.00 roots/explant, respectively (Fig. 5 and Table 3). The percent response of number of roots produced increased with increase in amount of NAA added up to the optimum level 2.0mg/L (Fig. 6).

Table 3. Rooting of *In vitro* raised shoots of bottle gourd (*Lagenaria siceraria*) with different concentrations of NAA

NAA concentration(mg/L)	Responding roots%	Average roots/ explant
0	0.00	0.00
0.5	32.08	1.67±0.33
1.0	50.94	2.67±0.33
1.5	56.60	3.67±0.33
2.0	100.00	5.33±0.33
2.5	50.94	2.67±0.33
3.0	32.08	1.67±0.33

Data are mean of three replicates with ± SE. Column do not differ significantly when compared by Duncan's MRT at 5% level of significance

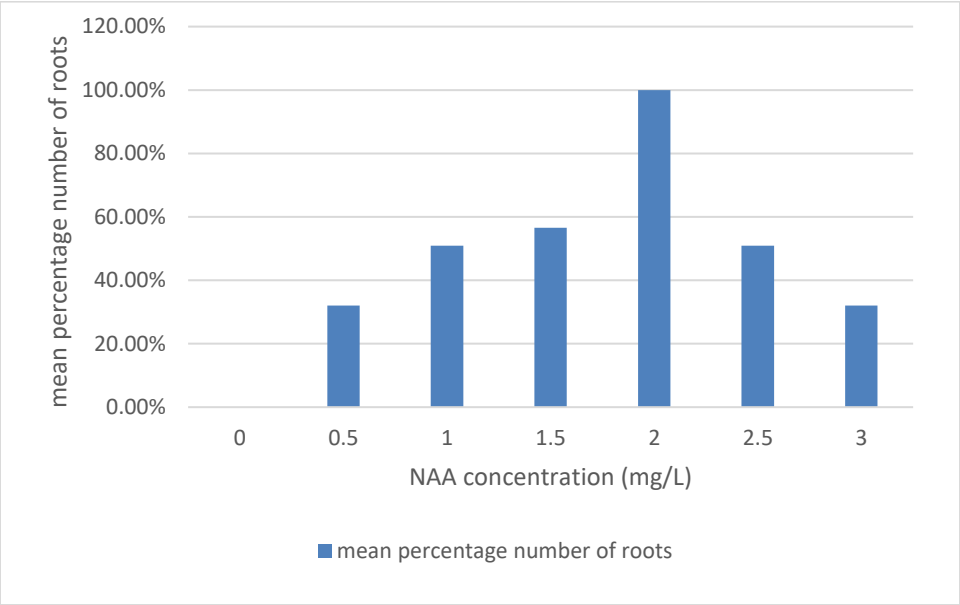

Fig. 5. A graph showing NAA concentration against percentage number of roots

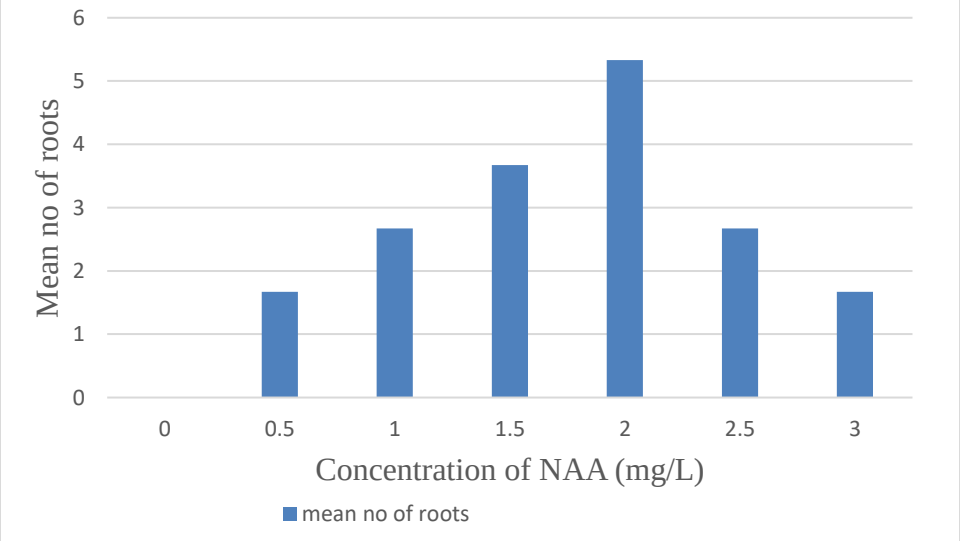

Fig. 6. Graph showing NAA concentration against average number of roots

3.2 Discussion

To completely eliminate microbial infection in tissue cultures, the formation of aseptic plant cultures necessitates a detailed understanding of the physiological condition of the plant as well as its susceptibilities to pathological pollutants. In this study, it was observed that the highest microbial contamination occurred in the control cultures (without sodium hypochlorite) when the various concentrations of the sterilant were evaluated. Fungal and bacterial contaminations are one of the most important limiting factors, particularly in medicinal plants during *in vitro* culture of explants" [11]. Hence, *in vitro* sterilization is a crucial stage in plant tissue culture, and the success of the sterilization process directly affects the culture's final outcomes" [12]. The effectiveness of the sterilizing step is crucial to the development of plant regeneration procedures and to the success of plant tissue culture [13]. "Inappropriate concentrations of sterilants have lethal effects on cell division and it restricts growth and development of explants. Therefore, suitable concentration, combinations and duration of exposure of sterilant is essential to raise *in vitro* cultures successfully" [14]. As surfaces of plant parts carry a wide range of microbial contaminants, the explants must be thoroughly surface-sterilized before planting them on the culture medium in order to avoid any source of infection. To disinfect explants, various sterilizing agents have been used such as hypochlorite solutions [15]. "The use of mercuric chloride and fungicides in explant surface sterilization is not recommended due to potential toxicity to both the researcher and the environment and furthermore, there is also an increased risk of plant growth retardation" [16]. In the current study, Sterilant at concentration 5% yielded maximum culture asepsis (100%). Higher sterilant treatments beyond the optimum level of 5% aggravated the Phyto-toxic effects leading to reduced explant. survival (0%). Similar results were recorded by [17] who recorded reduced explant survival under high treatments beyond 5% of sodium hypochlorite used and [18]. This research indicated that explants treated with lower concentration of sodium hypochlorite survived better than shoot tips treated with concentration of sterilant which may be due to increased phytotoxic effects due to high concentration of sterilant used. Shoot tips are immature, thin and tender. Tender explants show more phytotoxic effects of sterilant compared to mature ones and hence less survival when high amount of sterilant is used. Many scientists have also reported "a negative correlation with the high concentration of the disinfectants and the rate of explant viability" [19] and [13].

Application of BAP with MS media in *in vitro* culture provided the effective shoot regeneration and shoot numbers for *Lagenaria siceraria* when compared to the BAP-free treatment. The function of cytokinin is to effectively induce shoot regeneration for plantlet growth in plant tissue culture. Cytokinin, in particular BAP has been discovered to be one of the most effective cytokinin for shoot induction in plant tissue culture" [20]. The advantage of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily and also the ability of BAP to induce the production of natural hormones, such as zeatin within the tissue" [21]. Moreover, BAP is not easily metabolized and thus, persists in the medium in either free or ionized forms. Cytokinin is one of the most important factors in plant tissue culture especially in proliferation stage. It plays multiple roles in the plant development including cell division and cell expansion, plant protein synthesis stimulation and the activities of some enzymes" [22]. Use of cytokinin during multiplication stage increases the rate of proliferation as well as shoots quality" [23]. During this study MS medium supplemented with BAP (2 mg/L) was effective for 100% shoot multiplication in shoot tips of *Lagenaria siceraria*. The effect of BAP on multiple shoot formation has also been studied in various medicinal plant species such as *Stevia rebaudiana* [24] and *Ceropegia noorjahaniae* [25]. An increase of the concentration of BAP beyond the optimum concentration led to a decrease in the number of shoots and this corresponds with findings of Hasbullah and his colleagues [9]. In the present research, no significant increase in shoot multiplication of *Lagenaria siceraria* was found when the concentration of BAP was raised above 2.0mg/L. This finding was consistent with the report on that BAP concentration which was higher than the optimum level did not increase the shoot production rate of *Lantana camara* [9].

In order to succeed in micropropagation, high-quality adventitious roots must be produced [26]. Auxins are important factors involved in rooting because they promote adventitious roots formation in the vast majority of species [27]. Auxins are powerful cell division and differentiation regulators that support

adventitious root development in cuttings and micro shoots. Auxins regulate cell differentiation, starch hydrolysis, sugar and nutrient mobilization, cell differentiation, and rooting ability, resulting in a larger rooting percentage and more roots per rooted cutting [28]. For induction of adventitious rooting, higher auxin concentration is required [26].

In the present study 2.0mg/L of Naphthalene acetic acid (NAA) yielded maximum rooting percentage (100%) and highest number of roots (5.33per explant) compared to other concentration. which was similar to Hasbullah et al 2017 [9]. These results were also similar to Show kat Bhat et al 2022 [29] where highest ex vitro survival (89.67%) was observed in plantlets, which were rooted in MS media supplemented with IBA (2.0 mg/L). In the present study, no significant increase in root multiplication of *Lagenaria siceraria* was found when the concentration of NAA was raised above 2.0 mg/L which was the optimum concentration. This result was consistent with the report on Ash gourd that NAA concentration which was higher than the optimum level did not increase the shoot production rate [10]. This suggested that the best medium suitable for root induction in *L. camara* was the MS medium added with 2.0mg/L NAA. The report of Haquel [30] indicated that a low NAA concentration at 1.0mg/L produced 8.3 shoots per shoot tip explants of *Cucurbita maxima*. This suggested that the lower the concentration of NAA used, the higher the number of roots that were produced and a higher concentration of NAA should reasonably induce lower shoot numbers. This was in contrast to findings in the current study.

4. CONCLUSION

A novel regeneration protocol for the sterilization, multiple shoot production and *in vitro* rooting of an important medicinal plant, *Lagenaria siceraria* using shoot tip explants has been successfully developed. This was achieved using 5mg/L sodium hypochlorite that provided the most effective sterilization. 2.0mg/l of BAP provided the most effective multiple shoot shoot production and 2.0mg/l of NAA generated the highest number of roots per shoot and the highest root induction rate. The protocol described herein has the potential for rapid and mass propagation of *Lagenaria siceraria* for commercialization, conservation purposes and genetic improvements of this important medicinal plant.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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