*Biotechnology Journal International*

***18(2): 1-13, 2017; Article no.BJI.32715***
***Previously known as British Biotechnology Journal***
***ISSN: 2231–2927, NLM ID: 101616695***

# *In vitro* Propagation of *Oxytenanthera abyssinica* (A. Rich. Munro) from Seed Culture

**Birhanu Kahsay[1*], Firew Mekibib[2] and Adefris Teklewold[3]**

*[1]Collage of Natural and Computational Science, Debre Markos University, Debre Markos, Ethiopia.*
*[2]Collage of Agriculture and Environmental Science, Haramaya University, Dire Dawa, Ethiopia.*
*[3]CIMMYT, Addis Ababa, Ethiopia.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors BK and FM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors BK and AT managed the analyses of the study and the literature searches. Authors FM and AT reviewed the manuscript. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/BJI/2017/32715
Editor(s):
(1) Standardi Alvaro, (Rtd) Department of Agricultural and Environmental Sciences, University of Perugia, Italy.
Reviewers:
(1) Aysun Cavusoglu, Kocaeli University, Turkey.
(2) Dariusz Kulus, UTP University of Science and Technology, Poland.
Complete Peer review History: http://www.sciencedomain.org/review-history/19126

***Original Research Article***

***Received 10th March 2017***
***Accepted 26th April 2017***
***Published 18th May 2017***

## ABSTRACT

**Introduction:** In Ethiopia, *O. abyssinica* has varies economic importance. However, conventional propagation methods of *O. abyssinica* are generally inefficient due to their low multiplication rate, time consuming, labor intensive, and too costly.
**Aims:** The objective of this study was to develop a protocol for micropropagation of *O. abyssinica* through seed culture.
**Methodology:** For seed disinfection, NaOCl of 3, 4, and 5% concentration for 15, 20, and 25 min exposure time were tested. MS medium augmented with BAP or KN at different concentrations was used for shoot initiation and multiplication. For *in vitro* rooting, ½MS medium supplemented with IBA or NAA at different concentrations was used. Data were subjected to ANOVA and mean values were compared using LSD at a 5% of probability level.
**Results:** Seeds disinfected in 4.0% NaOCl for 25 minutes gave 71.6% clean explants and 23.45% germinated explants. In shoot initiation experiment all viable seeds were able to proliferate in 5-7

**Corresponding author: E-mail: birhanukahsay2017@gmail.com;*

days of culturing in all treatments; and 4.0 mg dm^-3 BAP was found better in maximum shoot initiation percent (86.67) and mean number of shoots per explants (4.8). Similarly, in shoot multiplication 4.0 mg dm^-3 BAP was effective in highest mean number of shoot (11.33) and multiplication rate (3.77). The maximum rooting percent (93.33) and maximum root number per clump (9.42) were found at 8.0 mg dm^-3 IBA. Finally, the survival rate of plantlets in greenhouse condition was found to be 91.67% after 30 days of acclimatization.

**Conclusion:** The study enables to develop an effective and applicable protocol for *O. abyssinica* micropropagtion.

*Keywords: Acclimatization; auxin; cytokinin; disinfection; micropropagation; rooting.*

## ABBREVIATIONS

*ANOVA : Analysis of Variance*
*BAP : 6-Benzylamino-purine*
*CRD : Completely Randomized Design*
*CV : Coefficient of Variation*
*DDW : Double Distilled Water*
*IBA : Indole-3-butyric acid*
*KN : Kinetin*
*LSD : Least Significant Difference*
*MS : Murashige and Skoog*
*NAA : Naphthalene acetic acid*
*PGRs : Plant Growth Regulators*

## 1. INTRODUCTION

Bamboo is hardened and fastest-growing perennial grass species [1] and it is a woody culms and gregarious, monocarpic flowering plant [2] belonging to the subfamily Bambusoideae under the family Poaceae [3]. About 43 species of bamboo distributed in 11 genera can be found in Africa, covering an estimated area of 3.6 million ha [4]. Out of these African bamboo species, Ethiopia has only two endemic species, namely the highland bamboo (*Yushania alpine* K. Schumach.) and lowland bamboo (*Oxytenanthera abyssinica* A.Rich. Munro) [5].

Bamboo is used for housing, handicrafts, pulp and paper industries, energy source, and food [4]. It has also great potential in reducing the risk of erosion [6] and high value in carbon sequestration [7]. Medical use of *O. abyssinica* is documented in different countries including Ethiopia [8]. *O. abyssinica* has also important phytochemicals with a resultant antioxidant property [9]. Furthermore, investigation on bamboo shoots showed that *O. abyssinica* shoot is rich in nutrients [10].

Conventionally, bamboos are propagated through seeds, clump division, rhizome and culm cuttings [11]. However, gregarious flowering at long intervals followed by the death of clumps, short viability of seeds [12], presence of diseases and some pests [13] are limiting factors to use seeds as valuable source of propagation. Vegetative propagation methods have limitation for mass propagation *i.e.* propagules are difficult to extract, bulky to transport, and planting materials are insufficient in number for large-scale plantation [14]. Considering problems encountered in both sexual and asexual conventional propagation of the *O. abyssinica* species, a method that brings about rapid large scale production of bamboo is highly desirable. In this regard different scholars recommended micropropagation as an excellent means to achieve this aim. The first tissue culture study on bamboo (*Dendrocalamus strictus*) was conducted by Alexander and Rao [15] who germinated embryos *in vitro*. Since then different researchers have been publishing scientific articles on successful micropropagation protocol through seed culture in different bamboo species; like, Arya et al. on *Dendrocalamus asper* [16], Arya et al. on *Dendrocalamus hamiltonii* [17], and Devi et al. on *Dendrocalamus giganteus* [18].

Nevertheless, their results show there is an interaction of species with hormonal types and levels included in the culture medium which necessitate the development/optimization of micropropagation protocols for every species under different conditions. And so far there is no report available on an efficient and reproducible protocol that can enable the *in vitro* rapid multiplication of *O. abyssinica* from seed culture. The main objective of this paper was, therefore, to develop a protocol for *in vitro* multiplication of *O. abyssinica* species from seed culture. The specific objectives for this study were to: determine explants-sterilization procedure, identify an appropriate cytokinin and determine its optimal concentration for shoot proliferation and multiplication; identify an appropriate auxin and determine its optimal concentration for root

induction; and assess acclimatization and survival rate of plantlets in greenhouse condition.

## 2. MATERIALS AND METHODS

### 2.1 Source of Experimental Material

The seeds for this study, collected before three weeks, were obtained from Asossa Agricultural Research Center Department of Forestry, Asossa, Benshangul Gumz Region, Ethiopia. Healthy seeds were selected carefully and they were stored in plastic bag at +4ºC in refrigerator. Seeds stored more than two months were not used.

### 2.2 Explants Surface Disinfection

The dehusked seeds soaked in distilled water for 2 h were washed by double distilled water (DDW) with liquid soap and followed by antifungal treatment with 0.25% (w/v) of Curzate® R WP (active ingredient of cupper) for 20 minutes. After pretreatment with 70% ethanol for 30 seconds under laminar air flow cabinet, they were then treated with three concentration levels (3%, 4% and 5% (w/v) active ingredient of chlorine) of locally produced bleach, sodium hypochlorite (NaOCl) for varying exposure times (15, 20, and 25 minutes). After decanting the sterilizing solutions, the seeds were washed three times each for five minutes with autoclaved distilled water to remove traces of NaOCl. For each sterilization treatments three jars, each with three seeds, were placed randomly in completely randomized design (CRD) arrangement. Data recorded from the sterilization experiment include the number of germinated (viable), contaminated, dead and clean cultures after 15 days of culturing.

### 2.3 Preparation of Stock Solutions

The nutrient media used were the Murashige and Skoog (MS) nutrient media components [19]. Stock solutions of major and minor salts, vitamins and plant growth regulators (PGRs) were prepared by dissolving the required quantity of chemicals in DDW. Stock solution of 6-Benzylaminopurine (BAP), Kinetin (KN), Naphthalene acetic acid (NAA), and Indole-3-butyric acid (IBA) were prepared by weighing and dissolving the powder in DDW at the ratio of 1mg:1ml and each PGR were dissolved by adding four to five drops 0.1N NaOH or 0.1N HCl based on the specific solvent requirement of the PGRs. Finally, after adjusting the final volume by adding DDW, the PGRs stock solutions were stored in refrigerator at +4ºC and they were only used before ten days of storage.

### 2.4 Culture Media Preparation

For all experiments the pH of the medium was adjusted to 5.80 before adding 0.4% agar. Full-strength MS medium fortified with 3% sucrose was used for disinfection, culture initiation and multiplication experiments, but half-strength MS medium fortified with same amount sucrose was used for root induction. About 30 ml of the medium were dispensed in each culture jar (200 ml) for disinfection and initiation experiment and 50 ml of the medium were dispensed in each culture jar (200 ml) for multiplication and rooting experiments. The media were autoclaved at 121ºC with 15PSi pressure for 20 minutes and kept under room temperature for three days before used.

### 2.5 Establishment of Culture Shoots

Disinfected seeds were cultured in 50 ml of MS medium containing varying level of BAP (0.5, 1.0, 2.0, 4.0 and 8.0 mg dm^-3). A separate experiment was set out with KN with 0.5, 1.0, 2.0, 4.0 and 8.0 mg dm^-3 concentration for shoot initiation study. MS medium without PGRs was used as control. A total of 11 treatments were employed and for each treatment five jars, each with three seeds, were used and placed in CRD arrangement on the growth room shelf with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, 40µmol m-2 s-1 irradiance) at 25 ± 2ºC. Number of days to shoot initiation and number of initiated seeds were recorded. Length of shoots, number of shoots/seed and shoot proliferation percentage were computed after 30 days.

### 2.6 Shoot Multiplication

To avoid the carry over effect of shoot initiation media during shoot multiplication, initiated propagules consisting of three shoots each were sub-cultured on PGRs free MS medium for two weeks. Each propagule was placed vertically and lightly pressed into the culture medium supplemented with varying levels of BAP (0.5, 1.0, 2.0, 4.0 and 8.0 mg dm^-3) and a separate experiment was set out with KN concentration of 0.5, 1.0, 2.0, 4.0 and 8.0 mg dm^-3. MS medium without PGRs was used as control. For each treatment five jars, each with three seeds, were used and kept under light conditions in CRD

arrangement. Data on number of shoot per explant, number of leaves per shoot, and shoots length (cm) were recorded after 6 weeks.

### 2.7 Rooting of Shoots

The *in vitro* regenerated shoots, three shoots in a bunch, were used for rooting studies after sub-cultured on PGRs free MS medium for 2 weeks. The rooting response of these shoots was studied on different concentrations of IBA (0.5, 1.0, 2.0, 4.0 and 8.0 mg dm^-3) and NAA (0.5, 1.0, 2.0, 4.0 and 8.0 mg dm^-3); and MS medium without PGRs was used as control. For each treatment five jars, each with three clumps, were used. All shoots were incubated on rooting medium for 4 weeks and kept under light conditions in culture room in CRD arrangement. Number of rooted clumps, number of roots per clumps, and average root length (cm) were recorded after 30 days.

### 2.8 Acclimatization

For hardening sixty *in vitro* well-rooted plantlets were planted into plastic bags filled with mixture of autoclaved soil (forest soil: sand: manure in 2:1:1 ratio). The transplanted plantlets were kept in greenhouse, which has an average temperature of 24±2ºC and relative humidity of 60-70%, under shade of polyethylene sheets and red cheese cloth for ten days. For the next five days the plantlets were acclimatized in an open greenhouse environmental condition with in the same room by removing the polyethylene sheet and red cheese cloth. Subsequently those plants that appeared harden and healthy were shifted to a warmer and drier open greenhouse room. After 15 and 30 days of transplanting, percentage of plantlets that were successfully acclimatized was recorded.

### 2.9 Data Analysis

The data was subjected to one-way analysis of variance (ANOVA) using the SAS software packages (version 9.1) and significant differences among mean values were compared using Least Significant Difference (LSD) at a 5% of probability level.

## 3. RESULTS AND DISCUSSION

### 3.1 Disinfection of the Explants

Among the different disinfectant treatments investigated, 3.0% NaOCl for 15 min recorded the highest contamination (100%). The lowest contamination (1.23%) was recorded from 5.00% NaOCl for 25 min duration. Table 1, the highest seed germination percentage (23.45%), the third minimum seed contamination percentage (4.93%), and 71.60% clean explants were recorded from 4% active chlorinated local bleach for 25 min. These results indicated that the chemical concentration and the duration at which the bamboo seeds were exposed to the disinfectant time were interrelated factors to obtain microorganism free explants and also affected the seed germination percentage significantly. The data also revealed (Table 1) that the contamination percentage was dramatically decreased as the exposure time increased within the same concentration of NaOCl solution. However, (Table 1) disinfection with higher concentration (5% w/v) of local bleaching solution for 25 minute resulted in least contamination and highest clean explants but no seed did germinate. This may be due to phytotoxic ability of NaOCl at longer exposure time; which lead to damage of explant tissues [20]. Wegayehu et al. [21] had also mentioned that high rate (71.43%) of peach node explants mortality was recorded from prolonged exposure of explants in high concentration of NaOCl (0.5% w/v). For an effective micropropagation protocol and other applications of *in vitro* cultures, explants must be disinfected at suitable disinfectant concentration for specified period where the explants can stay viable and contaminants free [22,23]. Therefore, in this experiment disinfection of bamboo seed with 4% (w/v) NaOCl solution for 25 min was the most effective disinfection treatment, which gave highest germination percentage; lowest contamination; and moderately clean explants.

### 3.2 Effect of BAP and KN on Establishment of Culture Shoots

As shown in Table 2, all viable seeds were able to proliferate shoots after 5-7 days of culturing in both control and cytokinin fortified MS medium. However, the initiation percentage, the days for initiation, number of shoots initiated, length of shoots, and leaves number were found to vary in the different concentrations of cytokinins and control treatment. The maximum shoot initiation percentage (86.67%) was recorded from 4.0 mg dm^-3 BAP supplemented MS medium; followed by 73.33% which was obtained from 2.0 mg dm^-3 KN augmented MS medium. The minimum shoot initiation percentage (Table 2) was 33.33% which was observed with seeds cultured at MS

**Table 1. Effect of NaOCl concentration and the exposure time on contamination, clean, and germination of explants *in vitro***

| NaOCl (local bleach) concentration (%) | Exposure time (min) | Contamination (%) | Clean explants (%) | Germination (%) |
|---|---|---|---|---|
| 3 | 15 | **100.00±0.00$^{a}$** | 0.00±0.00$^{h}$ | 0.00±0.00$^{c}$ |
| 3 | 20 | 85.18±5.55$^{b}$ | 3.70±5.55$^{gh}$ | 11.11±0.00$^{b}$ |
| 3 | 25 | 79.01±3.70$^{c}$ | 9.87±3.70$^{f}$ | 11.11±0.00$^{b}$ |
| 4 | 15 | 81.48±5.55$^{cb}$ | 6.17±5.58g$^{f}$ | 12.34±3.70$^{b}$ |
| 4 | 20 | 46.91±7.41$^{d}$ | 30.86±7.40$^{e}$ | **22.22±0.00$^{a}$** |
| 4 | 25 | 4.93±5.85$^{f}$ | 71.60±5.85$^{c}$ | **23.45±3.70$^{a}$** |
| 5 | 15 | 33.33±5.55$^{e}$ | 53.08±4.90$^{d}$ | 12.34±3.70$^{b}$ |
| 5 | 20 | 2.46±4.89$^{f}$ | 86.42±4.89$^{b}$ | 11.11±0.00$^{b}$ |
| 5 | 25 | 1.23±3.70$^{f}$ | **98.76±3.70$^{a}$** | 0.00±0.00$^{c}$ |
| CV% | | 10.54 | 12.61 | 18.55 |
| LSD | | 4.78 | 4.74 | 2.01 |

*Note: Means with the same latter (s) in the same column are not significantly different at P≤ .05 value using Fisher's LSD test. CV= Coefficient of Variation (%), LSD= Least Significant Difference, ± = Standard Deviation*

medium containing PGR free and 0.5 mg dm^-3 KN. This showed that the shoot inanition percentage from seed was greatly influenced by types and concentrations of cytokinin. Due to their ability enhancing in seed germination [24] and shoot initiation [25], investigations have been revealed that cytokinins were a key factor for bamboo species seed germination and multiple shoot proliferation [18]. The present study also found that, the ability to germinate and form multiple shoots of *O. abyssinica* seeds was dependent on the concentration and types of cytokinins. Culturing of seeds for more than 30 days in a medium resulted browning of shoots and consequently died up the whole plantlet.

Seeds cultured on PGRs free MS medium were the least in proliferation with a mean shoot number of 1.0±0.00 per seed. Of the various concentrations of cytokinins (BAP and KN) tried, highest shoot number (Table 2 and Figs. 1B and D) was obtained at 4.0 mg dm^-3 BAP (Fig. 1A) where 4.8±0.27 shoots per explant was formed after six weeks of culturing; followed by 3.46±0.02 shoots per seed and 3.00±0.00 shoots per seed obtained from 8.0 mg dm^-3 BAP and 4.0 mg dm^-3 KN fortified MS medium respectively.

Generally the present study indicated that the effect of BAP was better than KN in shoot proliferation percentage and multiple shoot induction. The present result is in agreement with the findings of other workers who have noted the effectiveness of BAP for the induction of multiple shoot from seeds in different bamboo species [26,27].

The longest (13.40 cm) and shortest (3.82 cm) shoots (Table 2) were recorded from PGRs free and 8.0 mg dm^-3 BAP fortified MS medium respectively. In both BAP and KN mean shoot length (Fig. 2) was negatively correlated with those cytokinins concentration level. This result might be due to seeds cultured on those medium gave less number of shoots, thus the nutritional competition was not happened strongly or due to the toxic effects of ethylene which can be produced at higher cytokinin concentration [28]. This is consistent with report of Arya et al. on decreasing of shoots height developed from seeds as cytokinin concentration increased [16].

Table 2 revealed that the maximum and minimum number of leaves (2.90 and 1.64) per shoot was recorded on control and 8.0 mg/l KN treatment respectively. From Table 2 we can also say that the number of leaves per shoot increases as the shoot length increases. This is in line with the finding of Saikia et al. [29] on *Bambusa nutans* bamboo species. Seeds cultured on MS medium without or with lower cytokinins developed 1-2 shoots per culture with roots (Fig. 1C), indicating that the seeds have enough optimum endogenous auxins to cytokinins ratio necessary to induce limited number of shoots and roots upon culturing. Seeds cultured at MS medium supplemented with higher BAP and KN concentration induce greater number of shoots but without roots. This is due to the imbalance between cytokinins to auxin ratio in which the increased level of cytokinins favors only shoot regeneration in the absence of equivalent auxin levels inside the plant.

A B C D

**Fig. 1. Effect of 4.0 mg dm^-3 BAP (A), 8.0 mg dm^-3 BAP (B), 0.5 mg dm^-3 KN (C), and 4.0 mg dm^-3 $^{3}$ KN (D) on seed germination and shoot formation from seed of *O. abyssinica* after 30 days of culturing**

**Table 2. Effect of BAP and Kinetin and their concentration on *in vitro* shoot initiation, shoot length, and leaf number from *O. abyssinica* seeds cultured on MS medium**

| Concentration of PGRs (mg per dm$^{-3}$) | | Shoot initiation % | Average day of initiation | Mean № of Shoots per explant | Mean Shoot length (cm) | Mean № of leave per shoot |
|---|---|---|---|---|---|---|
| BAP | KN | | | | | |
| 0 | 0 | 33.33±0.00$^{e}$ | 7.5±0.5$^{a}$ | 1.00±0.00$^{h}$ | **13.40±0.13$^{a}$** | **2.90±0.23$^{a}$** |
| 0.5 | 0 | 39.99±14.91$^{ed}$ | 7.4±0.54$^{a}$ | 2.00±0.00$^{f}$ | 5.90±0.10$^{c}$ | 2.51±0.18$^{cb}$ |
| 1 | 0 | 53.33±18.26$^{cd}$ | 7.26±0.65$^{a}$ | 2.00±0.00$^{f}$ | 5.14±0.14$^{ed}$ | 2.34±0.09$^{cbd}$ |
| 2 | 0 | 66.67±0.00$^{bc}$ | 6.5±0.5$^{b}$ | 2.20±0.27$^{fe}$ | 4.78±0.21$^{ef}$ | 2.16±0.07$^{ed}$ |
| 4 | 0 | **86.67±18.25$^{a}$** | **5.4±0.41$^{d}$** | **4.8±0.27$^{a}$** | 4.50± 0.55$^{f}$ | 1.93±0.24$^{fe}$ |
| 8 | 0 | 60.00±14.91$^{bc}$ | **6.0±0.61$^{bcd}$** | 3.46±0.02$^{b}$ | 3.82±0.22$^{g}$ | 1.93±0.17$^{fe}$ |
| 0 | 0.5 | 33.33±0.00$^{e}$ | 6.4±0.47$^{b}$ | 1.63±0.15$^{g}$ | **7.56±0.34$^{b}$** | **2.60±0.41$^{b}$** |
| 0 | 1 | 60.00±14.91$^{bc}$ | **6.10±0.74$^{bcd}$** | 2.23±0.22$^{e}$ | 5.40±0.14$^{d}$ | 2.29±0.19$^{cd}$ |
| 0 | 2 | **73.33±14.90$^{ab}$** | **5.5±0.5$^{d}$** | 2.50±0.00$^{d}$ | 5.34±0.32$^{d}$ | 1.99±0.12$^{fe}$ |
| 0 | 4 | 66.67±0.00$^{bc}$ | **5.6±0.41$^{cd}$** | 3.00±0.00$^{c}$ | 5.13±0.51$^{ed}$ | 1.76±0.14$^{fg}$ |
| 0 | 8 | 39.99±14.91$^{ed}$ | 6.23±0.47$^{cb}$ | 2.26±0.18$^{e}$ | 4.52±0.37$^{f}$ | 1.64±0.18$^{g}$ |
| CV (%) | | 22.8 | 8.83 | 6.23 | 6.49 | 9.50 |
| LSD | | 17.18 | 0.72 | 0.19 | 0.49 | 0.26 |

*Note: Means in the same column that are followed by different letters are significantly different (P ≤ .05) using Fisher's LSD test, LSD=Least Significant Difference, CV= coefficient of variation (%), ± = Standard Deviation*

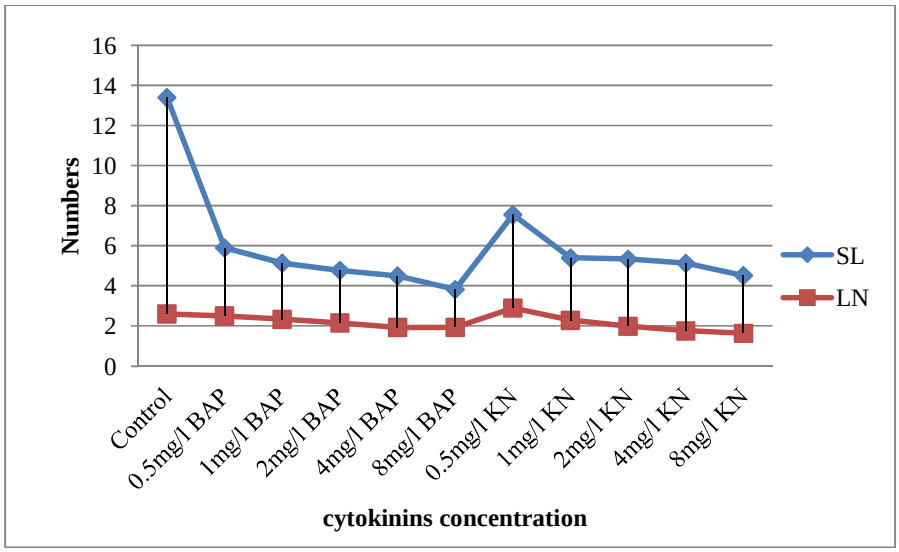


**Fig. 2. Effect of BAP and KN on seed shoots length (SL) and leaf number (LN)**

### 3.3 Effect of BAP and KN on Shoot Multiplication

Cytokinins were known to promote the function of other growth regulators like 2-isopentenyladenosine and zeatin [30]. In this study too (Table 3), the addition of either BAP or KN on most microshoots of *O. abyssinica* resulted in an increased multiplication rate and higher mean shoot number over PGRs free MS medium. Among the different concentration of BAP and KN investigated, 4.0 mg dm^-3 BAP showed maximum multiplication rate of 3.77±0.20, followed by 8.0 mg dm^-3 BAP (2.41±0.35). Lower rate of multiplication was recorded from 0.5 mg dm^-3 KN, 8.0 mg dm^-3 KN and PGRs free MS medium.

With regard to multiple shoot proliferation, as indicated in Table 3 and Figs. 4 A, B and C, 4.0 mg dm^-3 BAP displayed superiority over the other treatments with mean number of 11.33±0.62 shoots per propagule; followed by 8.0 mg dm^-3 and 2.0 mg dm^-3 BAP with 7.26±1.08 and 5.8±0.20, respectively. The lowest and not significantly different (Table 3) from control treatment mean number of shoot was obtained from 0.5 mg dm^-3 and 8.0 mg dm^-3 KN treatments. The average superiority of BAP over KN in shoot induction may due to its ability to induce production of natural hormones such as zeatin within the tissues than other synthetic cytokinins [31]. Accordingly, the ability of plant tissues to metabolize the natural hormones is faster than artificial growth regulators. The effect of BAP in inducing multiple shoots has already been reported in bamboo species like *Arundinaria callosa* [32] and *Bambusa oldhamii* [33]. Interestingly, the synergistic effect of BAP and KN for increased shoot multiplication rate and proliferation was also reported on *Bambusa tulda* and *Melocanna baccifera* [34].

In both cytokinins (BAP and KN) the shoot length is inversely related to their respective concentration (Fig. 3). The longest shoot length (3.92±0.30) (Table 3) was recorded from PGRs free MS medium, followed by 3.66±0.20 and 3.4±0.14 from 0.5 mg dm^-3 BAP and 0.5 mg dm^-3 KN, respectively. The shortest shoot length 2.62±0.20, 2.64±0.32, and 2.65±0.19 (Table 3) were recorded from higher concentration of cytokinins: 8.0 mg dm^-3 BAP, 8.0 mg dm^-3 KN and 4.0 mg dm^-3 KN, respectively. This finding is in line with report of Shroti et al. [35]. According to Woeste et al. [28] the reduction in shoot length at higher concentration of BAP and KN might be due to the toxic effects of ethylene produced at higher cytokinin concentration.

As revealed in Table 3, the maximum leaf number per shoot (1.86) was recorded on PGRs free media followed by 1.41 and 1.34 in 0.5 mg dm^-3 BAP and 0.5 mg dm^-3 KN supplemented MS medium, respectively. On decreasing the concentration of cytokinins, the number of shoots decreased but the leaf number and shoot length were increased (Fig. 3). These findings are in agreement with what was reported by Saikia et al. [29] on *B. nutans in vitro* propagation. As *O. abyssinica* is woody plant [14], the occurrence of phenol exudation at the cut ends of explants was the main problem faced during the multiplication study (Fig. 4 D). This phenolic exudation delayed the time required for sub-culturing accompanied with gradual browning of the shoots leaf and medium that eventually ends up in death of the

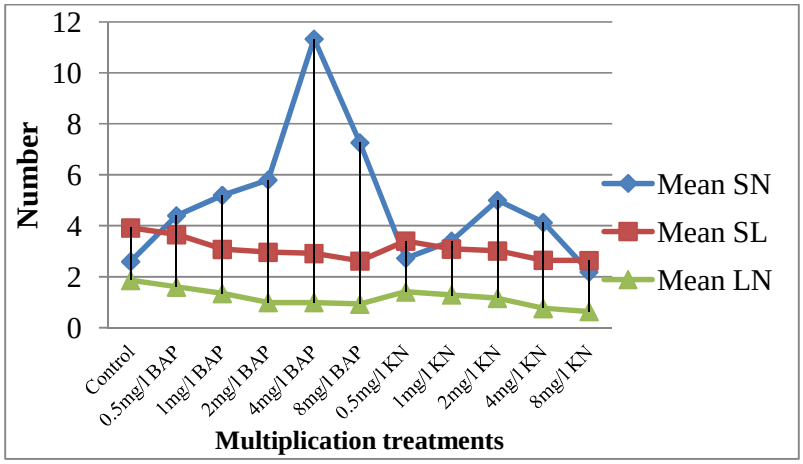


**Fig. 3. Effect of BAP and KN on shoot number (SN), shoots length (SL), and leaf number (SL)**

A B C D

**Fig. 4. Effect of 2.0 mg dm^-3 BAP (A), 4.0 mg dm^-3 BAP (B), 2.0 mg dm^-3 BAP (C) on *in vitro* multiplication of *O. abyssinica* microshoots after 4 weeks of culturing. (D) Dried microshoots and browning of medium due to phenol exudation**

**Table 3. Effect of BAP and Kinetin concentration in MS medium on *in vitro* shoot propagation of *O. abyssinica***

| Concentration of PGRs (mg per $dm^{-3}$) | | Mean № of shoots | Mean of shoots length (cm) | Mean № of leaves per shoot | Multiplication rate |
|---|---|---|---|---|---|
| BAP | Kin | | | | |
| 0 | 0 | 2.6±0.24$^{g}$ | **3.92±0.30$^{a}$** | **1.86±0.05$^{a}$** | 0.86±0.08$^{g}$ |
| 0.5 | 0 | 4.4±0.29$^{e}$ | **3.66±0.20$^{ab}$** | **1.60±0.13$^{b}$** | 1.46±0.09$^{e}$ |
| 1 | 0 | 5.2±0.12$^{d}$ | 3.08±0.08$^{c}$ | 1.34±0.09$^{c}$ | 1.73±0.03$^{d}$ |
| 2 | 0 | 5.8±0.20$^{c}$ | 2.96±0.13$^{c}$ | 0.99±0.05$^{e}$ | 1.93±0.06$^{c}$ |
| 4 | 0 | **11.33±0.62$^{a}$** | 2.92±0.13$^{c}$ | 0.99±0.11$^{e}$ | **3.77±0.20$^{a}$** |
| 8 | 0 | 7.26±1.08$^{b}$ | 2.62±0.20$^{d}$ | 0.93±0.10$^{e}$ | 2.41±0.35$^{b}$ |
| 0 | 0.5 | 2.73±0.27$^{g}$ | 3.4±0.14$^{b}$ | 1.41±0.11$^{c}$ | 0.91±0.09$^{g}$ |
| 0 | 1 | 3.40±0.36$^{f}$ | 3.09±0.21$^{c}$ | 1.29±0.08$^{cd}$ | 1.13±0.12$^{f}$ |
| 0 | 2 | 5.0±0.23$^{d}$ | 3.02±0.24$^{c}$ | 1.16±0.07$^{d}$ | 1.66±0.07$^{d}$ |
| 0 | 4 | 4.13±0.29$^{e}$ | 2.65±0.19$^{d}$ | 0.76±0.14$^{f}$ | 1.37±0.09$^{e}$ |
| 0 | 8 | 2.19±0.18$^{g}$ | 2.64±0.32$^{d}$ | 0.64±0.12$^{f}$ | 0.73±0.05$^{g}$ |
| CV (%) | | 9.03 | 6.77 | 8.89 | 8.99 |
| LSD | | 0.56 | 0.26 | 0.13 | 0.18 |

*Note: Means in the same column that are followed by different letters are significantly different at P≤ .05 using Fisher's LSD test, LSD=Least Significant Difference CV= coefficient of variation (%), ± = Standard Deviation*

explants without any response. However, this problem was overcome by proper removal of brown leaf sheaths and recurrent transfer of explant to fresh medium every 2-3 weeks while multiplying shoots. To prevent browning, similar technique was also recommended by Sharma and Sarma [36].

### 3.4 Effect of IBA and NAA on Root Induction

The development of healthy root system is required for the successful establishment of *in vitro* regenerated shoots to adapt the external environments. Therefore, in the present study the potential of IBA and NAA on rooting of *O. abyssinica* microshoots separately was investigated for drawing possible recommendation in future large scale use. Accordingly, the ANOVA showed that type and concentration of auxins had highly significant effect (P< 0.001) on rooting percentage, root number and root length of *O. abyssinica in vitro* rooting. In most of the treatments rooting induction was started after 10 day culturing but in higher auxins concentration (8.0 mg dm^-3) it was delayed up to 15 day culturing.

Among different concentrations of IBA and NAA investigated (Table 4), the treatments formed by adding 4.0 and 8.0 mg dm^-3 of IBA to the ½ strength MS showed equal per cent of root induction (93.33±14.90), followed by ½ strength MS + 2.0 mg dm^-3 IBA (66.67 %) and ½ strength MS + mg dm^-3 NAA (66.67 %). The other treatments: ½ strength MS fortified with 0.5 mg dm^-3 IBA (39.99%), 1.0 mg dm^-3 IBA (46.66%), 0.5 mg/l NAA (39.99%), 1.0 mg dm^-3 NAA (53.33%), 4.0 mg dm^-3 NAA (46.66%), and 8.0 mg dm^-3 NAA (39.99%) showed poor

response similar to the PGRs free treatment (Table 4, Fig. 5). Generally the average percent of root induction was higher for the IBA than NAA treatments. Superiority of IBA over NAA for *in vitro* root induction was also reported by Parthiban et al. and Diab and Mohamed [37,38].

With regard to average number of roots per clump, ½ strength MS + 8.0 mg dm^-3 IBA registered its superiority over the other treatments by exhibiting 9.42±0.08 root number per clump (Fig. 5C). The 2nd (4.72±0.15) (Fig. 5 B) and 3rd (4.50±0.00) (Fig. 5 D) highest number of roots per clump, which were not significantly different from each other, were obtained by fortifying ½ strength MS medium with 4.0 mg dm^-3 IBA and 2.0 mg dm^-3 NAA, respectively. Table 4 revealed that the other level of concentrations showed lower mean number of root but significantly different from the control. As reported by Parthiban et al. [37] the best root number for *Bambusa balcooa* was obtained from higher IBA concentration supplemented MS medium. In present study, 8.0 mg dm^-3 IBA was found to be superior in both rooting percent and number of roots produced, which is in agreement with reports of Parthiban et al. and Diab and Mohamed [37,38].

Perusal of Table 4 reveals that the longest root of 8.90±0.49 cm was obtained from PGRs free ½MS medium, followed by 0.5 and 1.0 mg dm^-3 IBA fortified ½ strength medium with respective mean length of 7.48±0.21 and 7.02±0.23 cm. The shortest mean root length (1.21±0.21 cm) was obtained from 8.0 mg dm^-3 NAA fortified ½MS medium. Generally, the mean root length decreased as auxins concentration increased. Comparing the two auxins, IBA supplemented ½MS medium showed better efficiency in favoring root growth than NAA. The positive effect of IBA alone on rooting of *in vitro*

**Table 4. Effect of IBA and NAA on *in vitro* rooting of *O. abyssinica* microshoots at ½ strength MS medium**

| Concentration of PGRs (mg per dm$^{-3}$) | | Mean of rooting percentage | Mean № of roots | Mean of roots length (cm) |
|---|---|---|---|---|
| IBA | NAA | | | |
| 0 | 0 | 33.33±0.00$^{c}$ | 1.20±0.44$^{g}$ | **8.90±0.49$^{a}$** |
| 0.5 | 0 | 39.99±14.9$^{c}$ | 1.80±0.44$^{f}$ | **7.48±0.21$^{b}$** |
| 1 | 0 | 46.66±18.2$^{c}$ | 2.18±0.32$^{f}$ | 7.02±0.23$^{c}$ |
| 2 | 0 | 66.67±0.00$^{b}$ | 3.80±0.44$^{de}$ | 5.73±0.09$^{d}$ |
| 4 | 0 | **93.33±14.9$^{a}$** | 4.72±0.15$^{b}$ | 5.69±0.16$^{d}$ |
| 8 | 0 | **93.33±14.9$^{a}$** | **9.42±0.08$^{a}$** | 4.26±0.07$^{e}$ |
| 0 | 0.5 | 39.99±14.9$^{c}$ | 4.10±0.22$^{cd}$ | 3.34±0.12$^{f}$ |
| 0 | 1 | 53.33±18.2$^{bc}$ | 4.20±0.27$^{cd}$ | 3.12±0.11$^{f}$ |
| 0 | 2 | 66.67±0.00$^{b}$ | 4.50±0.00$^{bc}$ | 2.31±0.20$^{g}$ |
| 0 | 4 | 46.66±18.26$^{c}$ | 3.40±0.41$^{e}$ | 2.18±0.06$^{g}$ |
| 0 | 8 | 39.99±14.91$^{c}$ | 2.10± 0.22$^{f}$ | 1.21±0.21$^{h}$ |
| CV (%) | | 24.58 | 8.34 | 4.53 |
| LSD | | 17.66 | 0.40 | 0.26 |

*Note: Means in the same column that are followed by different letters are significantly different at P≤ .05 using Fisher's LSD test, LSD=Least Significant Difference, CV= Coefficient of Variation (%), ± = Standard Deviation.*

**A** **B** **C** **D**

**Fig. 5. Effect of 4.0 mg dm^-3 IBA (B), 8.0 mg dm^-3 IBA (C), and 2.0 mg dm^-3 (D) NAA on *in vitro* rooting of *O. abyssinica* microshoots after four weeks of culturing; (A) microshoots cultured on PGRs free ½MS medium for rooting**

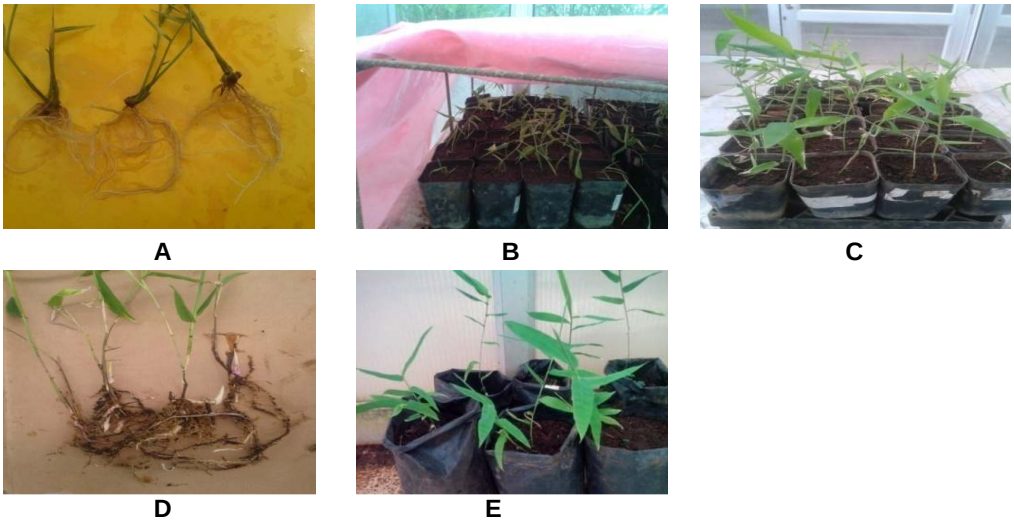


**Fig. 6. *In vitro* rooted plantlets during transplanting (A), polyethylene sheet and cheese close covered pantalets after 3 day transfer (B), plantlets after 30 days acclimatization (C) well rooted plantlets after 30 days of acclimatization (D), and plantlets after 45 days acclimatization (E)**

propagated plantlets had been already established in *Bambusa Bambos* and *D. giganteus* [18,39].

Differential effectiveness among auxins might be attributed to the concentration of free auxin that reached the target competent cells, and the metabolic stability of the auxins. As discussed by Caboche et al. [40] an inhibitory and toxic effect of NAA at above optimum concentration was also observed. Due to its stability than NAA and convertibility to natural indole-3-acetic acid in plant tissues enable IBA to sustain auxin longer and enhance rooting in the culture media than NAA [41].

### 3.5 Acclimatization

After 15 and 30 day of hardening, the survival rate of *in vitro* rooted (Fig. 6A) plantlets was 95% and 91.67%, respectively. In the present study, during hardening stage some plantlets were found wilting in the first three days of transferring (Fig. 6B) and some leaves were dried up and subsequently detached from the shoots. This may be due to unrestricted loss of water from their leaves or low hydraulic conductivity of roots and root-stem connections [42]. However, after ten day of hardening two-three new leaves were developed from each shoot. Gradually the plantlets started growing and the leaf number increased as the plant height increases. Progressively (Figs. 6C, E), as the acclimatization process continue, color of the leaves turned to deep green and size of the leave get increased with the size of the plant. Furthermore as showed in Fig. 6 D, proliferations of new tillers were observed after 30 days of hardening.

## 4. CONCLUSIONS

4% active chlorinated local bleach for 25 min was effective in disinfection experiment. 4.0 mg dm^-3 BAP supplemented MS medium showed highest shoot proliferation percentage (86.67%), the highest shoot number (4.8), and requires 5.4 average days to induce shoot. Similarly, for the shoot multiplication experiment, among the tested cytokinin, BAP at 4.0 mg dm^-3 gave the highest shoot number (11.33) and shoot multiplication rate (3.77%). In the root induction IBA was superior to NAA. The 8.0 mg dm^-3 IBA supplemented ½MS medium gave the maximum root number, 9.42 roots per clump and the highest percent of root induction (93.33%). PGRs free ½MS medium induced longest root. Ultimately, hardening well rooted plantlets grown in pots filled with sterilized forest soil under greenhouse condition for 10 days assured a survival rate of as much as 92% after 30 days in the open greenhouse environments.

## ACKNOWLEDGEMENTS

The authors would like to thank the Haramaya University for financing and Holeta Agricultural Research Center, Agricultural Biotechnology Laboratory for allowing and facilitating to work in their laboratory.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. BPG (Bamboo Phylogeny Group).. An updated tribal and subtribal classification of the bamboos (Poaceae: Bambusoideae). In: Proceedings of the 9th World Bamboo Congress. Antwerp, Belgium: World Bamboo Organization. 2012;3-27.
2. Singh SR, Singh R, Kalia S, Dalal S, Dhawan AK, Kalia RK. Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo-a plant with extraordinary qualities. Physiology and Molecular Biology of Plants. 2013;9(1):21-41.
3. GPWG (Grass Phylogeny Working Group). Phylogeny and subfamilial classification of the grasses (Poaceae). Annals of the Missouri Botanical Garden. 2001;88:373-457.
4. FAO (Food and Agriculture Organization). Global forest resources assessment. Main report. FAO Forestry Paper 163. Food and Agriculture Organization of the United Nations, Rome, Italy; 2010.
5. Seyoum K. Anatomical characteristics of Ethiopian low land bamboo (*Oxytenanthera abyssinia*). Presentation of the International Network for Bamboo and Rattan (INBAR). Beijing, China; 2008.
6. Yenesew A, Yihenew GS, Belayneh A. Farmers' perception on high land bamboo (*Yushania alpina*) for land resource conservation in Banja district, northwestern Ethiopia. Wudpecker Journal of Agricultural Research. 2014;3(1):1-9.
7. Yiping L, Yanxia L, Henley KB, Guomo Z. Bamboo and climate change mitigation: a comparative analysis of carbon sequestration. International Network for Bamboo and Rattan (INBR), Technical Report. 2010;32.
8. Hall JB, Inada T. *Oxytenanthera abyssinica* (A.Rich. Munro). *In*: Louppe, D., Oteng-Amoako, A.A. & Brink, M. (Editors). Prota 7(1): Timbers/Bois d'œuvre 1. [CD-Rom] PROTA, Wageningen, Netherland; 2008.
9. Bartholomew IO, Maxwell EI. Phytochemical constituents and *in vitro* antioxidant capacity of methanolic leaf extract of *Oxytenanthera abyssinica* (A.Rich Murno) determined at varying concentrations of the plant extract. European Journal of Medicinal Plants. 2013;3(2):206-217.
10. Sisay F.. Site factor on nutritional content of *Arundinaria alpine* and *Oxytenanthera abyssinica* bamboo shoots in Ethiopia. Journal of Horticulture and Forestry. 2013;5(9):115 121.
11. Banik RL. Selection criteria and population enhancement of priority bamboos. INBAR Technical Report N°5 Genetic enhancement of bamboo and rattan INBAR New Delhi. 1995;99-110.
12. Bereket H. Study on establishment of bamboo processing plants in Amhara Regional State. M.Sc. Thesis Addis Ababa University, Addis Ababa, Ethiopia; 2008.
13. Demelash A, Zebene T, Yared K. Effect of storage media and storage time on germination and field emergence of *Oxytenanthera abyssinica* seeds. International Journal of Basic and Applied Sciences. 2012;1(3):218-226.
14. Kassahun E, Weih M, Ledin S, Christersson L. Biomass and nutrient distribution in a highland bamboo forest in southwest Ethiopia: Implications for management. Forest Ecology and Management. 2005;204:159-169.
15. Alexander MP, Rao TC. *In vitro* culture of bamboo embryo. Current Science. 1968;37:415.
16. Arya S, Sharma S, Kaur R, Arya ID. Micropropagation of *Dendrocalamus asper* by shoot proliferation using seeds. Plant Cell Report. 1999;18:879-882.
17. Arya ID, Kaur B, Arya S. Rapid and mass propagation of economically important bamboo *Dendrocalamus hamiltonii*. Indian Journal of Energy. 2012;1(1):11-16.
18. Devi WS, Bengyella L, Sharma GJ. *In vitro* seed germination and micropropagation of edible bamboo *Dendrocalamus giganteus* Munro using seeds. Biotechnology. 2012;1-7.
19. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with

tobacco tissue cultures. Physiol Plant. 1962;15:473-97.
20. Kulus D. Micropropagation of *Kalanchoe tubiflora* (Harvey) Hamet. Nauka, Przyroda, Technologie. 2015;9(1):1-8.
21. Wegayehu F, Firew M, Belayneh A. Optimization of explants surface sterilization condition for field grown peach (*Prunus persica* L. Batsch. Cv. Garnem) intended for in vitro culture. African Journal of Biotechnology. 2015;14(8):657-660.
22. Oyebanji OB, Nweke O, Odebunmin NB, Galadima NB, Idris MS, Nnod UN, Afolabi AS, Ogbadu GH. Simple, effective and economical explant surface sterilization protocol for cowpea, rice and sorghum seeds. African Journal of Biotechnology. 2009;8(20):5395-5399.
23. Teixeira da Silva, Jaime A, Kulus D, Zhang X, Zeng S, Ma G, Piqueras A. Disinfection of explants for saffron (*Crocus sativus* L.) tissue culture. Environmental and Experimental Biology. 2016;14(4):183-198.
24. Miransaria M, Smith DL. Plant hormones and seed germination: Review. Environmental and Experimental Botany. 2014;99:110-121.
25. Ashraf MF, Aziz M, Kemat N, Ismail I. Effect of cytokinin types, concentrations and their interactions on *in vitro* shoot regeneration of *Chlorophytum borivilianum* Sant. & Fernandez. Electronic Journal of Biotechnology. 2014;EJBT-00047:1-5. Available:http://dx.doi.org/10.1016/j.ejbt.2014.08.004 Accessed on September 16, 2014
26. Nadgir AL, Phadke CH, Gupta PK, Parsharami VA, Nair S, Mascarenhas AF. Rapid multiplication of bamboo by tissue culture. Silvae Genetica. 1984;33(6):219-223.
27. Tuan TT, Tu HL, Giap DD, Du TX. The increase in *in vitro* shoot multiplication rate of *Dendrocalamus asper* (Schult. f.) Back. ex Heyne. TẠP CHÍ SINH HỌC. 2012;34(3se):257-264.
28. Woeste KE, Vogel JP, Kieber JJ. Factors regulating ethylene biosynthesis in etiolated *Arabidopsis thaliana* seedlings. Physiologia Plantarum. 1999;105:478-484.
29. Saikia SP, Mudoi KD, Borthakur M. Effect of nodal positions, seasonal variations, and shoot clump and growth regulators on micropropagation of commercially important bamboo, *Bambusa nutans* Wall. Ex. Munro. African Journal of Biotechnology. 2014;13(9):1961-1972.
30. Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA. Plant hormones and plant growth regulators in plant tissue culture: Review. *In vitro* Cellular and Developmental Biology Plant. 1996;32: 272-28.
31. Zaerr JB, Mapes MO. Action of growth regulators. *In*: Bonga, J.M. Durzan, D.J. (Eds.). Tissue Culture in Forestry. Martinus Nijhoff/Dr. W. Junk Publisher, The Hague/Boston/London. 1982;231-255.
32. Devi WS, Sharma GJ. *In vitro* propagation of *Arundinaria callosa* Munro- an edible bamboo from nodal explants of mature plants. The Open Plant Science Journal. 2009;3:35 39.
33. Thiruvengadam M, Rekha KT, Chung IM. Rapid *in vitro* micropropagation of *Bambusa oldhamii* Munro. The Philippine Agricultural Scientist. 2011;94(1):1-8.
34. Waikhom SD, Louis B. An effective protocol for micropropagation of edible bamboo species (*Bambusa tulda* and *Melocanna baccifera*) through nodal culture. Hindawi Publishing Corporatione, Scientific World Journal. 2014, Article ID 345794:1-8.
35. Shroti RK, Upadhyay R, Niratkar C, Singh M. Micropropagation of *Dandrocalamus asper* through inter nodal segment. Bulletin of Environment, Pharmacology and Life Sciences. 2012;1(3):58-60.
36. Sharma P, Sarma KP. *In vitro* propagation of *Bambusa balcooa* for a better environment. International Conference on Advances in Biotechnology and Pharmaceutical Sciences Bangkok Dec. 2011;248-252.
37. Parthiban KT, Kanna SU, Kamala K, Vennila S, Durairasu P. *In vitro* organogenesis and rhizogenesis of thornless bamboo (*Bambusa balcooa*). Journal of International Academic Research for Multidisciplinary. 2013;1(11): 401-413.
38. Diab EE, Mohamed SE. *In vitro* morphogenesis and plant regeneration of bamboos (*Oxytenanthera abyssinica* A. Rich. Munro). International Journal of Sustainable Crop Production. 2008;3(6): 72-79.
39. Anand M, Brar J. *In vitro* propagation of an edible bamboo *Bambusa bambos* and assessment of clonal fidelity through molecular markers. Journal of Medical and Bioengineering. 2013;2(4):257-261.

40. Caboche M, Muller J, Chanut F, Aranda G, Iirakoglu S. Comparison of the growth promoting activities and toxicities of various auxin analogs on cells derived from wild type and a non-rooting mutant of tobacco. Plant Physiology. 1987;83:795-800.
41. Strader LC, Bartel B. Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid: Review Article. Molecular Plant. 2011;4(3):477–486.
42. Kumar K, Rao IU. Morphophysiologicals problems in acclimatization of micropropagated plants in *ex-vitro* conditions-A Reviews. Journal of Ornamental and Horticultural Plants. 2012;2(4):271-283.



*Peer-review history:*
*The peer review history for this paper can be accessed here:*
*http://sciencedomain.org/review-history/19126*