

Develop Micro clonal -propagation protocol for *Oxytenanthera abyssinica* A.Rich. Munro to large scale micro-propagation

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ABSTRACT

In Ethiopia, *Oxytenanthera abyssinica* A.Rich. Munro has various 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. The objective of this study was to develop a protocol for mass micropropagation of *O. abyssinica* through seed culture. Murashige and Skoog (MS) medium augmented with 6-Benzylaminopurine (BAP) was used for shoot initiation and multiplication. For in vitro rooting, MS medium supplemented with 3-Indole-butyric acid (IBA) was used.

In shoot initiation experiment all viable seeds were proliferated in 5-7 days of culturing. In shoot multiplication at 0.004 g/L BAP was successfully shoot multiplied, also best root responding were found at 0.005 g/l IBA.

The present optimized protocol enables for any actors who need large numbers of low land bamboo seedling for industry, small and micro enterprise or for reforestation programs.

Keywords: IBA; BAP; micropropagation; rooting.

1. INTRODUCTION

Bamboo is a hardy and fastest-growing perennial grass species [1] and it is a woody culm and gregarious, monocarpic flowering plant [2]. They belong to the subfamily Bambusoideae and family Poaceae (sometimes called Gramineae), in the same family with cereal crops such as rice and wheat and sugar cane [3]. The term bamboo comprises more than 1,500 species that are widely distributed in the tropical, subtropical and temperate regions of all continents except Antarctica and Europe, between 46°N and 47°S. Geographically bamboo distribution can be classified into three zones: the Asian Pacific zone, the American zone and the African zone [4]. The highest diversity and area coverage of bamboo is recorded from the Asian continent, followed by America and Africa [5]. The sizes of bamboos vary from small annuals to giant perennial timber bamboo species [6]. Dwarf bamboos may be as little as 10cm in height, but stands of tall species may attain 15–20m, and the largest known (e.g. *Dendrocalamus giganteus* and *Dendrocalamus brandisii*) grow up to 40m in height and 30cm in culm (stem) diameter [7; 8; 9].

43 species of bamboo in 11 genera can be found in Africa, covering an estimated area of 3.6 million ha [10]. Out of these African bamboo species, Ethiopia has only two endemic species, namely the highland bamboo (*Yushania alpina* K. Schumacher) and lowland bamboo (*Oxytenanthera abyssinica* A. Rich. Munro). These two species are restricted to limited agro-ecological regions, i.e. in highland areas of altitude 2400–3500 m.a.s.l. and in lowland areas from 500–1800 m.a.s.l. [11].

For adaptation, Ethiopia has imported different bamboo species and they are under field trial in different locations those are: *Dendrocalamus asper*, *Dendrocalamus hamiltonii*, *Dendrocalamus giganteus*, *Dendrocalamus membranaceus* Munro, *Bambusa vulgaris* Var. *green*, *Bambusa vulgaris* Var. *Vitata*, *Guadua amplexifolia* [12].

Bamboo is used for housing, handicrafts, pulp and paper industries, energy source, and food . It has also high value in carbon sequestration [13]. Medical use of *O. abyssinica* is documented in different countries including Ethiopia [14]. *O. abyssinica* has also important phytochemicals with a resultant antioxidant property [15]. Furthermore, investigation on bamboo shoots showed that *O. abyssinica* shoot is rich in nutrients [16].

Conventionally, bamboos are propagated through seeds, clump division, rhizome and culm cuttings [17]. However, gregarious flowering at long intervals followed by the death of clumps, short viability of seeds [18], presence of diseases and some pests [19] are limiting factors to use seeds as valuable source of propagation.

Even vegetative propagation methods have limitation for mass propagation since propagules are difficult to extract, bulky to transport, and planting materials are insufficient in number for large-scale plantation [20]. Considering problems encountered in both sexual and asexual conventional propagation of the *O. abyssinica* species, innovative 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 [21] 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* [22], Arya et al. on *Dendrocalamus hamiltonii* [23], and Devi et al. on *Dendrocalamus giganteus* [24]. 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 also , Kahsay et al.; 2017 for *O. abyssinica* species developed protocol for mass propagation from seed culture by using 3-BAP, NAA and IBA hormone at different concentration 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 using 3-BAP and IBA hormone for better improvements of Kahsay *et al.*; 2017. The specific objectives for this study were to determine, 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.

2. MATERIALS AND METHODS

2.1 Source of Experimental Material

The seeds for this study were obtained from Bahradr Environment and Forest Research center, Ethiopia. Healthy seeds were selected carefully and they were stored in plastic bag at +4°C in refrigerator. Seeds were stored more than a year in Bahradr Tissue culture laboratory.



Figure 1. Seeds of *Oxytenanthera abyssinica* Figure 2. selected healthy seed

2.2 Explants Surface Disinfection

Selected healthy seeds as shown in **figure 2** were sterilized to get rid of all micro-organisms. Also, the seeds were washed with tap water to remove debris. Then, to get clean seeds it soaked in distilled water for 2 hrs by shaking and washed by double distilled water (DDW) with liquid soap with 2-3 drops of Tween -20 for 20 minutes. Then, treated by antifungal of mancozine 20 g/l for 20 minute and washed the seeds with DDW three times. By following those procedure, seeds were treated by 2% of NaOCl for

20 minute and washed the seeds three times by 2-3 drop of Tween- 20 for five minute . After pre-treatment,the seeds were treated with 1% NaOCl and washed three times by DDw. Finally, it treated with 70% ethanol for 30 seconds under laminar air flow cabinet. After sterilization of the MS medium ,for shoot initiation three jars for each treatments (0.003 ,0.004 and 0.005 g/L BAP) five seeds were placed randomly in completely randomized design (CRD) arrangement.

2.3. Preparation of Stock Solutions for initiation, multiplication and rooting

<u>Macro-nutrients 1</u>	<u>Componet mg/L</u>
1.NH ₄ NO ₃	1650
2.KNO ₃	1900
3.KH ₂ PO ₄	170
4.MgSO ₄ .7H ₂ O	370
<u>Macro-nutrients 2</u>	<u>Componet mg/L</u>
CaCl ₂ .2H ₂ O	440
<u>Micro- nutrients 1</u>	<u>Componet mg/L</u>
1.MnSo ₄ .4H ₂ O	22.3
2.ZnSO ₄ .7H ₂ O	8.6
3. H ₃ BO ₃	6.2
4.KI	0.83
<u>Micro-nutrients 2</u>	<u>Componet mg/L</u>
1.Na ₂ MO ₄ .2H ₂ O	0.25
2.CUSO ₄ .5H ₂ O	0.25
3.COCl ₂ .6H ₂ O	0.025
<u>Iron source</u>	<u>Componet g/L</u>
1. Na ₂ EDTA	0.0378 g
2.FeSO ₄ .7H ₂ O	0.0278 g
<u>Vitamins1</u>	<u>Componet mg/L</u>
1. myo-inositol	100
<u>Vitamin 2</u>	<u>Componet mg/L</u>

1.Thiamine-HCl	1
2.Pyridoxine-HCl	0.5
3.Nicotinic -acid	0.5
4.Glycine	2
<u>Carbon source</u>	
1. Sucrose	30 g/L
<u>Gelling agent</u>	
Plant agar powder	4g/L
PH	5.8
<u>Additional chemicals for phenol exudation</u>	

1. Activated charcoal ----- 0.1 g/l

The above Stock solutions of major and minor salts , vitamins and plant growth regulators (PGRs) of 6-Benzylaminopurine (BAP) acid and Indole-3-butyric (IBA) were used by dissolving in distilled water.

Finally, after adjusting the final volume ,the hormone stock solutions were stored in refrigerator at +4°C and other nutrient medium were kept at room temperature and used after 5 days from preparation time for targeted experiment.

2.4 Culture Media Preparation

For all experiments the pH of the above mentioned prepared nutrient medium was adjusted to 5.80 before adding 0.4% agar. Full-strength MS medium with 3% sucrose was used for culture initiation and multiplication experiments.About 50 ml of the medium were dispensed to 300 ml jar for initiation experiment and also 50 ml of the medium were dispensed to 300 ml jar for multiplication and rooting experiments. The media were autoclaved at 121°C with 15PSi pressure for 20 minutes and kept under room temperature for four days before used.

2.5. Establishment of Culture Shoots

Disinfected seeds were cultured in 9 jars that contained the above mentioned nutrient 50 ml of MS medium with BAP and 3 jars PGRs free medium for

shoot initiation study. Then the cultured samples were brought to growth room shelf with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, $40\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance) at $25 \pm 2^\circ\text{C}$. After seven days, all of the cultured samples were initiated shoot.

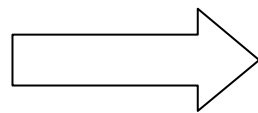


Figure 3. Cultured seed

Figure 4. Initiated leaf from cultured seed

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 0.003-0.005 g/litre of BAP with each activated charcoal for inhibition of oxidants of the cells mostly for phenol exudation. MS medium without PGRs was used as control. 12 jars each with three propagules were used and kept under light conditions. Then, after two weeks multiplication of new leaf were best at 0.004 g/litre of BAP as it showed in **figure 5**.



Figure 5. Multiplication of new leaf from initiated seed at 0.004 g /L BAP hormone.

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 (0.004, 0.005 and 0.006 g/L) and with for each treatments used 0.1 g/L activated charcoal for inhibition of oxidants of the cells mostly for phenol exudation on MS medium and without hormone was used as control. For each treatment three 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. Among all treatment, 0.005 g/L of IBA solution experiment were best for root formation as it shown in **figure 7**.



Figure 6. Root formation of Low land bamboo via IBA hormone after a week from single shoot.



Figure 7. Root formation of Low land bamboo via IBA hormone after one month.

3. Results and discussion

3.1 Disinfection of the explants

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 [25,26]. Therefore, in this experiment disinfection of bamboo seed were under took by 2 % (w/v) NaOCl solution for 25 min, 2-3 drops of Tween -20 for 20 minutes and antifungal of 20 g/l for 20 minute were the most effective

disinfection treatment, which gave highest germination percentage; lowest contamination; and moderately clean explants.

3.2 Effect of BAP on establishment of culture shoots

In this experiment all viable seeds were proliferated 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 vary in the different concentrations of cytokinins and control treatment. The best shoot initiation was recorded from 0.005 g/L 3- BAP supplemented in a MS medium. This showed that the shoot initiation percentage from seed was greatly influenced by types and concentrations of cytokinin. Their ability in this study for enhancing seed germination [27] and shoot initiation [28] is lined in this authors, investigations have been revealed that cytokinins were a key factor for bamboo species seed germination and multiple shoot proliferation [29]. Culturing of seeds for more than 30 days in a medium resulted browning of shoots and consequently died up the whole plantlet. Generally the present study indicated that the effect of 0.005 g /L BAP was best for shoot proliferation percentage and multiple shoot induction. The is research 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 [30,31]. The longest (13.9 cm) and shortest (3.3 cm) shoots were recorded from 0.005 g/L -3 BAP and PGRs free fortified MS medium respectivel. Seeds cultured at MS medium supplemented from the present treatment higher BAP concentration induce greater number of shoots but without roots. This is due to the inapprporiate balance between cytokinins to auxin ratio in which the high level of cytokinins favors only shoot regeneration in the absence of equivalent auxin levels inside the bamboo plant.

3.3 Effect of BAP on shoot multiplication

Cytokinins were known to promote the function of other growth regulators like 2-isopentenyladenosine and zeatin [32]. In this study too ,the addition of BAP on most microshoots of *O. abyssinica* resulted in an increased multiplication

rate and higher mean shoot number over PGRs free MS medium. Investigated BAP at 0.005 g/L showed a good multiplication rate. The effect of BAP in inducing multiple shoots has already been reported in bamboo species like *Arundinaria callosa* [33] and *Bambusa oldhamii* [34]. Interestingly, the synergistic effect of BAP and KN for increased shoot multiplication rate and proliferation was also reported on *Bambusa tulda* and *Melocanna baccifera* [35]. The occurrence of phenol exudation at the cut ends of explants was the main problem faced during the multiplication study as shown in **Figure 8**. 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. The IBA was found nice in both rooting percent and number of roots produced, which is in agreement with reports of Parthiban et al. and Diab and Mohamed [36,37].



Figure 8. Browning of bamboo leaf due to phenol release.

4. Conclusions and recommendation

2 % of NaOCl solution for 25 min, 2-3 drops of Tween -20 for 20 minutes and antifungal of mancozine of 20 g/l for 20 minute were effective for disinfection of low land bamboo seed .0.004 g/L -3 BAP supplementedwith MS medium showed best shoot proliferation, better shoot number and requires 5 -7 days to induce shoot. Similarly, for the shoot multiplication experiment, the tested cytokinin at 0.004 g/L BAP gave the efficient shoot number and shoot multiplication .In the root induction, IBA was also best at 0.005 g/L supplemented with MS medium gave best root number.

Finally,this study recommends to use this protocol for mass propagation of low land bamboo for reafforestation of degraded land which is highly exsposed for drought and for industry purpose.

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6. References

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