Micro clonal propagation protocol for Lowland bamboo (*Oxytenanthera abyssinica*) and adapted the propagated seedling in the dryland areas of Abay Valley, Ethiopia

Abstract

Oxytenanthera abyssinica has various economic and ecological benefits in Ethiopia. Nowadays, the need for planting the species across the wider landscape, as plantation forest, is increasing. However, conventional propagation methods of *O. abyssinica* are found generally to be inefficient because they have low multiplication rate, are time consuming, labor intensive and too costly. Accordingly, tissue culture protocol was developed at Bahir Dar Tissue Culture Laboratory during 2021/22 for production of sufficient number of lowland bamboo seedlings through seed culture. Murashige and Skoog (MS) medium augmented with 6-Benzylaminopurine (BAP) was used for shoot initiation and multiplication. MS medium supplemented with 3-Indole –butric acid (IBA) was used for in vitro rooting. In shoot initiation experiment all viable seeds were proliferated in 5-7 days of culturing. Shoot was successfully multiplied at 0.004 g/L BAP and best rooting response was found at 0.005 g/l IBA. The propagated seedling was planted and adapted in the lowland areas of Abay valley at 1000 m asl. The present optimized protocol and adaptation of the propagated seedling in the dryland areas enable actors who need large numbers of low land bamboo seedling for aforestation/reforestation programs.

Key words: **Lowland Bamboo**;**Micro propagation**; **Tissue culture; 6-Benzylaminopurine; Murashige and Skoog medium (MS**); **Rooting**

1. Introduction

Bamboo is a perennial grass belonging to the Poaceae (Gramineae) family and Bambuseae subfamily. Bamboos have a long history as an exceptionally versatile and widely used resource in the world. Nowadays, it is becoming so increasingly important in the world's forest economy, because it is: superior wood substitute, cheap and efficient to produce and utilize, environmentally friendly and the world forest is shrinking hence requiring alternative sources[1]. Bamboos are fastest-growing plants in the world, reaching 100 cm height per day [2] and one of the most important nontimber forest resources or a potential alternative to wood and wood products [3].

Bamboo is widely distributed in Asia, Latin America, and Africa from sea level to highlands in tropical, subtropical, and temperate countries [4, 5]. According to the world bamboo resources assessment report, Ethiopia, Kenya, and Uganda possess most of the bamboo resources in Africa [6]. Ethiopia contributes to the leading coverage constituting more than 1.44 million hectares [7]. This constitutes about 86% of the total area of bamboo on the continent and 7% of the world [8].

There are two indigenous bamboo species in Ethiopia namely lowland bamboo (*Oxytenanthera abyssinica* A. Rich. Munro) and Ethiopian highland bamboo (*Arundinaria alpina* K. Schumach) These two species are found restricted in limited agro ecological regions, i.e. highland bamboo in highland areas of altitude 2200-3500 ma.s.l. and lowland bamboo from 500-1800 ma.s.l [9]

Ethiopian lowland bamboo is used for housing, handicrafts, pulp and paper industries, energy source, and food; it fits ISO standards for the production of an array of lumber-based and stick-based products. Medical use of *O. abyssinica* is documented in different countries including Ethiopia. O. abyssinica has also important phytochemicals with a resultant antioxidant property). Furthermore, investigation on bamboo shoots showed that *O. abyssinica* shoot is rich in nutrients [10].

O. abyssinica is a perennial monocarpic or once flowering plant. Conventionally, it is propagated through seeds, rhizome-based and clump division techniques. However, gregarious flowering at long intervals followed by the death of clumps, short viability of seeds presence of diseases and some pests are limiting factors to use seeds as valuable source of propagation. The rhizome-based offset method, is inefficient as propagules are difficult to extract, bulky to transport, have low multiplication rate, labor intensive and too costly and planting materials are insufficient in number for large-scale plantation. Considering problems encountered in both sexual and asexual conventional propagation of *O. abyssinica*, innovative method that brings about rapid large scale production is highly desirable.
In this regard micro propagation is an excellent means developed protocol for mass propagation of *O. abyssinica* at Holeta Biotechnology Lab 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 [11].

Thus, the main objective of this research was to further optimize the protocol for *in vitro* multiplication of *O. abyssinica*, from seed culture using 3-BAP and IBA hormones for better improvements at Bahir Dar Tissue Culture Laboratory. The specific objectives were to: 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 planting the propagated seedling in the dryland areas of Abay valley for restorations of the degraded lands of the valley to protect the Great Ethiopian Renaissance Dam from siltation.

2. Materials and Methods

2.1 Source of Experimental Material

The seeds for this study were obtained from Assosa, Benishangul Gumuz Region, Ethiopia. Healthy seeds were selected carefully and they were stored in plastic bag at +4°C in refrigerator. Seeds were stored for more than a year in Bahradar Tissue Culture laboratory.

2.2 Explants Surface Disinfection

The selected healthy seeds were sterilized to remove all microorganisms. The seeds were also washed with tap water to remove dirt. To obtain clean seeds, they were soaked in distilled water for 2 h with shaking and washed in Double Distilled Water (DDW) with liquid soap and 2–3 drops of Tween -20 for 20 min. The seeds were then treated with 20 g/L of Manitol antifungal for 20 minutes and the seeds were washed three times with DDW. Following this step, seeds were treated with 2% NaOCl for 20 min and washed three times with 2–3 drops of Tween-20 for 5 min. After pretreatment, seeds were treated with 1% NaOCl and washed three times with DDw. Finally, it was treated with 70% ethanol for 30 s in a laminar flow cabinet. After sterilization of MS medium, five seeds were randomly placed in three jars for each treatment (0.003, 0.004, and 0.005 g/L BAP) in a Completely Randomized Design arrangement (CRD) for germination initiation.

2.3. Culture Media Preparation

For all experiments, the pH of the nutrient medium prepared above was adjusted to 5.80 before adding 0.4% agar. Full strength MS medium containing 3% sucrose was used for culture initiation and growth experiments. Approximately 50 ml of medium was added to the 300 ml jar for initiation experiments, and 50 ml of medium was also added to the 300 ml jar for propagation and rooting experiments. The medium was autoclaved at 121°C and 15 PSi pressure for 20 min and stored at room temperature for 4 days before use.

2.4. Establishment of Culture Shoots

Disinfected seeds were cultured in nine jars for germination initiation studies, including 50 mL of MS medium with the above nutrients, BAP, and three jars of medium without plant growth regulators (PGR). The culture samples were then incubated in a culture room with a light/dark 16/8 h photoperiod at 25 ± 2° using cool white fluorescent lighting (photon flux density, 40 µmol m^{-2} s^{-1} irradiance. After seven days, all the cultured samples initiated shoots.

2.5. 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/L BAP with each activated charcoal for inhibition of oxidants

of the cells mostly for phenol exudation. MS medium without PGRs was used as control. Twelve (12) jars each with three propagules were used and kept under light conditions. After two weeks multiplication period, new leaves were best obtained in the 0.004 g/L BAP treatment as shown in Figure 1 (left).

2.6. Rooting of Shoots

The *in vitro* regenerated three shoots in bunch were used for rooting studies after sub-culturing 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). For each treatment 0.1 g/L activated charcoal was used for inhibition of oxidants of the cells and for phenol exudation on MS medium and without hormone 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 treatments, 0.005 g/L of IBA solution experiment were best for root formation as shown in Figure2

Figure 1 (left). Initiated leaf from cultured low land bamboo seed; (right) Multiplication of new leaf from initiated seed at 0.004 g /L BAP hormone

Figure 2. Root formation of Low land bamboo via IBA hormone after one month.

Figure 3. Acclimatizing bamboo seedling in nursery

3. Results and Discusión

3.1 Disinfection of the explants

To develop effective micropropagation protocols and other applications of in vitro culture, explants must be treated with an appropriate concentration of disinfectant to ensure that they remain viable and free of contaminants [12,13]. Therefore, in this experiment, bamboo seeds were disinfected with 2% (w/v) NaOCl solution for 25 min, 2-3 drops of Tween-20 for 20 min, and 20 g/L antifungal agent for 20 min. was the most effective. effective. Disinfection treatment resulting in the highest germination rate, minimal contamination and reasonably clean explants.

3.2 Effect of BAP on the establishment of cultured buds

In this experiment, all viable seeds were buds that multiplied after 5–7 days of culture on both control and cytokinin-rich MS media. However, the rate of initiation, number of days to initiation, number of shoots initiated, shoot length and number of leaves were found to vary depending on the concentration of cytokinin and control treatment. The best shoot initiation was recorded when MS medium was supplemented with 0.005 g/L 3-BAP. This showed that the rate of shoot formation from seeds was strongly influenced by the type and concentration of cytokinin. Their ability to improve seed germination [14] and shoot formation[15] in this study is highlighted by the authors. Studies have shown that cytokinins are important factors in seed germination and multi-shoot multiplication of bamboo species [16] . If seeds are grown in a medium for more than 30 days, the shoots will turn brown and the entire plantlet will die. In general, this study suggested that the effect of 0.005 g/L BAP was the best for the rate of shoot proliferation and the induction of multiple shoots. The results of this study are consistent with the findings of other researchers who discovered the effectiveness of BAP in inducing multiple buds from seeds of various bamboo species [17,18]. The longest (13.9 cm) and shortest (3.3 cm) shoots were recorded from MS medium supplemented with 0.005 g/L-3 BAP and MS medium without PGR, respectively. However, when seeds were cultured in MS medium supplemented with a high concentration of BAP using this treatment, many shoots without roots were generated. This is due to an inappropriate balance between cytokinin and auxin, with high levels of cytokinin favoring shoot regeneration only when corresponding levels of auxin are absent in bamboo plants. 3.3 Effect of his BAP on shoot proliferation Cytokinin is known to promote the function of other growth regulators such as 2-Isopenteny l Adenosine and Zeatin [19]. In this study, we also added his BAP to the micro shoots of most O. abyssinica led to increased reproduction. Higher average bacterial counts compared to MS medium without PGR. BAP

tested at 0.005 g/L showed good growth rate. The effect of BAP in inducing multiple shoots has already been reported in bamboo species such as *Arundinaria callosa* [20] and *Marmora oldhamii* [21]. Interestingly, the synergistic effect of BAP and KN on increasing shoot proliferation rate and proliferation was also reported in *Marmora turda* and *Melocanna bacifera* [22].

The occurrence of phenol exudation at the cut ends of explants was the main problem faced during the multiplication study as showed in Figure 4. 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[23,24]. As shown in Figure 6, the appearance of phenolic exudates at the cut ends of the explants was the main problem during the growth study.

Figure 4. Browing of bamboo leaf due to phenol release.

This phenol immersion delays the time required for subculturing leading to gradual browning of the leaf and shoot medium and eventually leading to death of. IBA was found to be better in terms of both rooting rate and number of roots produced.

3.3 Geo-Spatial features of the adaptation sites of the propagated seedling

One of the adaptation sites in Abay valley, Ethiopia is topographically steep sloppy, rocky and found close to 1080 m asl. Similarly, also the other site is mainly flat terrain to slightly sloppy having clay soil at elevation of 1650 m asl. Both sites are poor in terms of their vegetation cover, and are vulnerable to soil erosion. Among, the planted seedlings 90% were survive in two sited after assessing at six months. So saving these sites from further degradation would have an

important implication in minimizing siltation going to the river Abay and consequently to GERD

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Figure 5. The adapted low land bamboo seedling at Abay Valley.

4. Conclusions

The 2% NaOCl solution for 25 minutes, the 2-3 drops of Tween-20 for 20 minutes, and the Antifungal Agent Mancogin 20 g/L for 20 minutes are effective for disinfection of lowland bamboo seeds.

The BAP at 0.004 g/L, supplemented with Murashige and Skoog medium (MS) was effective and showed the best bud proliferation, the best number of shoots, and required 5 to 7 days for shoot induction.

Similarly, in shoot proliferation experiments, cytokinins tested at 0.004 g/L 6-Benzyl Amino Purine (BAP) resulted in efficient shoot numbers and shoot proliferation.

The 3-Indole –Butyric Acid (IBA) was also optimal for root induction at 0.005 g/L supplemented with MS medium gave the best root numbers.

Finally, this study recommends the use of this protocol for mass propagation of lowland-grown bamboo for reforestation of degraded lands highly exposed to drought.

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