PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF THE ETHIOPIAN LOWLAND BAMBOO (*Oxytenanthera abyssinica* (A. Rich)) THROUGH NODAL CULTURE

M.Sc. Thesis

ASHENAFI GEBRELIBANOS KIFLU

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Protocol Optimization for *In Vitro* Propagation of the Ethiopian Lowland Bamboo (*Oxytenanthera abyssinica*, A. Rich) through Nodal Culture

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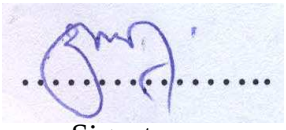
APPROVAL SHEET
SCHOOL OF GRADUATE STUDIES
JIMMA UNIVERSITY

As thesis research advisors, we hereby certify that we have read and evaluated the thesis prepared under our direction, by Ashenafi Gebrelibanos, entitled **(Protocol Optimization for *In Vitro* Propagation of the Ethiopian Lowland Bamboo (*Oxytenanthera abyssinica*, A. Rich) through Nodal Culture).** We recommend that, it will be accepted as fulfilling the thesis requirement for the Degree of Master of Science in Plant Biotechnology.

Belayneh Admassu (Ph.D)		……..……
Major Advisor	Signature	Date
Kassahun Bantte_ (Ph.D)	……	…….
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DEDICATION

This work is dedicated to my father, Ato Gebrelibanos Kiflu Gebremichael, who firstly guided me the road to school and passed away during the Ethio-Eritrean war without seeing my achievements.

STATEMENT OF THE AUTHOR

I , Ashenafi Gebrelibanos , hereby declare that the work presented in the thesis manuscript entitled, **"Protocol Optimization for *In Vitro* Propagation of the Ethiopian Lowland Bamboo (*Oxytenanthera abyssinica,* A. Rich) through Nodal Culture"** for partial fulfillment of the requirements for the award of the Degree of Masters of Science in Plant Biotechnology at Jimma University is an authentic record of my own work, under the supervision of my major advisor Dr. Belayneh Admassu and my co-advisor, Dr. Kassahun Bantte.

Duly acknowledging all source materials I used in this thesis, I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. It can be deposited at the University Library to be made available to users under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the School of Graduate Studies when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

Name: Ashenafi Gebrelibanos Signature: _____

Place: Jimma University

Date: / /2012

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BIOGRAPHICAL SKETCH

The author, Mr. Ashenafi Gebrelibanos Kiflu, was born from his father Ato Gebrelibanos Kiflu Gebremichael and his mother W/ro Hadas Gebremeskel Mihret on February 4, 1987 in Adwa, Central Zone of Tigray Regional State, Northern Ethiopia. He attended his elementary education from 1994-1999 in Unamerad primary school and his junior elementary school in Mai-tsadik elementary school from 2000-2001. After successfully passing the national exam of the regional state, he joined Nigist Saba Comprehensive Secondary School in 2002 and completed high school as well as preparatory education in natural sciences in 2005.

Mr. Ashenafi joined Ambo University in Dec. 2005 and graduated with B.Sc. in Plant Sciences with great distinction in July, 2008. After graduation, he worked as graduate assistant at Debre Berhan University (Nov. 2008 – Aug. 2009) and starting from Sept. 2009, he served up to the rank of assistant lecturer at Aksum University. He joined Jimma University in Sept. 2010 to persue his M.Sc. in Plant Biotechnology under the sponsor of the current employer, Aksum University.

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ABBREVIATIONS

2-ip	2- isopentenyl-adenine
ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
CRD	Completely Randomized Design
EABP	East African Bamboo Project
GA_3	Gibberellic Acid
IAA	Indol Acetic Acid
IBA	Indol-3-Butyric Acid
Kin	Kinetin
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PGRs	Plant Growth Regulators
PTC	Plant Tissue Culture
SNNP	Southern Nations, Nationalities and Peoples of Ethiopia

PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF THE ETHIOPIAN LOWLAND BAMBOO (*Oxytenanthera abyssinica* (A. Rich)) THROUGH NODAL CULTURE

ABSTRACT

The Ethiopian lowland bamboo, (Oxytenanthera abyssinica, A. Rich), is an important plant in both rural and urban areas of Ethiopia having myriad of uses ranging from construction, furniture and handicrafts to food, bioenergy and medicinal values. The plant flowers once after many decades and dies soon after flowering. The resulting genetically variable seeds abort soon after fertilization and fail to sustain the generation due to short viability. Other vegetative propagation methods pose difficulty in establishment as well as management and more terribly count their ages from their mother plants and die shortly. Hence, micropropagation from seed grown mother plants is one of the way outs to overcome this problem. The present study was initiated at the National Plant Biotechnology Laboratory in order to optimize in vitro protocol for mass propagation through nodal culture from one year old bamboo plants grown in a glasshouse. The experiments were laid out in a completely randomized design with 30 observational units per single treatment. MS basal medium was used for all experiments. After efficacious sterilization that resulted up to 100% initiation without contamination, explants inoculated in the medium supplemented with 2.5mgl $^{-1}$ BAP resulted in 100% of initiated explants within four days. This treatment showed other extra shoots of independent auxiliary buds sprouting from a single explant that could incur effective signs of multiplication during initiation. For in vitro multiplication, initiated shoots were cut in to lengths of 2 cm and treated with different concentrations and combinations of BAP and Kinetin. In the 1st and 2nd stages of multiplication, MS+2.5mgl $^{-1}$ BAP +1.5mgl $^{-1}$ kinetin resulted in higher number of shoots per explant (5.90 and 5.84, respectively). However, MS+1.5mgl $^{-1}$ BAP +1.5mgl $^{-1}$ kinetin resulted in higher number of shoots per explants in the 3rd and 4th stages. In terms of multiple nodes per shoot, PGR free medium was the best of all the treatments. For root induction, shoots inoculated on full strength hormone free medium developed roots (having length of 7.3cm,>3 main roots and 36.7% rooting efficiency). Addition of wide range concentrations of IBA (0.25-45mgl $^{-1}$) resulted in drying of shoots without rooting. With the exception of very high level of IBA concentration (50mgl $^{-1}$), which showed little signs of forced rooting, all rooting hormone treatments failed to initiate rooting. Remarkably, straight acclimatization of the remaining percentage of shoots which failed to root in PGR free medium and subsequent establishment by ex vitro rooting resulted in a survival rate of 43.33%, which increased the percentage of acclimatized plantlets at the same time. Hence, in addition to the 83.3% rate of acclimatization obtained from the in vitro rooted shoots, the overall acclimatization efficiency reached an overall survival rate of 63.3%. Therefore, utilization of this protocol helps to generate planting materials in large scale and in short period of time at fair cost.

Key Words: Bamboo, *ex vitro* rooting, micropropagation, *Oxytenanthera abyssinica*.

1. INTRODUCTION

Bamboo, a common name for about 45 genera and about 1500 species, represents perennial, woody usually shrubby or treelike plants of the grass family (Poaceae) and Bambuseae subfamily. The main stem of the aboveground part of the plant is the culm, while the underground part constitutes the rhizome and root system. Once the rhizome-root system is well established, new bamboo shoots attain full height (6-8 m) and diameter (4-8 cm) within 2-3 month (Ensermu *et al.*, 2000). The bamboo plant flowers towards the end of its lifetime (14 to 120 years in some species) and then dies soon after. For this reason, bamboo flowering is considered as a "disease" by Ethiopians who live in the bamboo growing areas. Ethiopia has two bamboo species: the lowland bamboo (*Oxytenanthera abyssinica* (A Rich)) that constitutes 85% and the highland bamboo (*Yushania alpine* (K. Schum)) that covers the remaining 15% (Kassahun, 2003).

Bamboo plants grow in tropical and temperate regions of the world, being more abundant in the former, particularly in Southeast Asia. According to Ohrnberger (1999), bamboo species cover more than 14 million hectares of land, 4% of forest plantations. Out of these species, Africa possesses about 43 species on over 1.5 million ha of land (Kigomo, 1988). Ethiopia has over one million hectares of bamboo resources which corresponds to two thirds of all Africa's current resources in the sector and 7% of the world total. Currently, our bamboo populations are in danger due to drastic drying after subsequent flowering that could devastate the whole plant ecosystem without regenerating in the next generation (Kassahun, 2003).

In this era, where activity of human kind brings a major threat for the environment, it's indispensable to cultivate plants that could have substantial roles for human welfare by sustaining the environment and protecting climate change. For almost half of the human race in the world, life would be quite different without bamboo (Ramanuja *et al.*, 2008). Bamboo is an economically important multipurpose plant. It is a source for food, fodder, furniture, building materials, paper, particle board, energy, and medicine. It also plays a vital role in environmental amelioration, biodiversity preservation, soil conservation and waste purification (Rao, 2000). Bamboo has an important role to play in restoring balance

to the planet's climate system and reducing global warming. Bamboo absorbs 35% more carbon dioxide compared to trees. It also emits 35% more oxygen (Segundino, 2010). The potential that bamboo products offer to promote income generation at national and local levels has long been known amongst stakeholders in many of the countries of the world.

In Ethiopia, the use of bamboo is restricted to construction, furniture and household utensils. Although bamboo is not an integral part of the Ethiopian economy, it plays a very important role socially, economically and ecologically in areas where it occurs naturally as well as where it is planted or used (Ensermu *et al.*, 2000). A number of tasks are being carried out to use the huge bamboo resource in Ethiopia and to get access to local and international markets for bamboo products (Arsema, 2008).

Recently, bamboo products manufacturing workshops are expanding in big cities of the country. The regional governments and city council's micro and small enterprises development offices as well as Forest Products Utilization Research Center are now attempting to promote the development of small-scale enterprise such as bamboo, wood and metal sectors that do not require sophisticated technology. The development of bamboo entrepreneurship business in Ethiopia has a promising prospect in the future. For example, the number of small-scale bamboo workshops had increased from 10 to 58 within 10 years in Addis Ababa alone. Currently in Addis Ababa and other big cities modern types of bamboo furniture such as sofa chairs, beds, tables, shelves, lampshades and fruit trays are available in the market. Ethiopian traditional restaurants and modern recreation centers or hotels of high standard are furnished with attractive bamboo made products are marvelous places for cultural celebration and tourist enjoyment in many cities (Arsema, 2008).

Studies of the bamboo sectors in Ethiopia and Kenya during 1999-2001, funded by INBAR and local institutions and conducted by national experts, have identified a wide range of differing production-to-consumption systems, and pinpointed possible interventions that would promote the production and trade of bamboo products (http://www.eabp.org.et/). There is high local and international demand for industrial

bamboo production. Yet, product diversification and value addition are very limited in the local bamboo industry. Knowledge on the potential of the various possible products is still poor, particularly with regard to industrial goods and as an alternative material in construction. Know-how transfer for skills development, access to appropriate technology and standards is needed. Altogether, potentials of bamboo production for local construction and international markets remain still untapped. Most bamboo is harvested from naturally occurring stands with minimal conservation or reforestation. Many species of bamboo are endangered because of harvesting and the lack of knowledge on propagation methods and flowering control (most species die after flowering) (Kassahun, 2003).

In most bamboo species, seed set bamboo is an infrequent event on which, even if it occurs, there is a need to wait from 10 up to 120 years. It is not only the deferment, what makes it more worse and devastating is that, seed setting in most bamboos is followed by total death of all the plants, which might havoc the whole ecosystem and lives dependent on the plant. In the case of Ethiopian species, flowering in lowland bamboo seems to be more frequent than the highland one. According to the local communities in the areas of bamboo production in lowlands of Ethiopia, it flowers every 30-35 years. These phenomena were observed in the lowland bamboo forest of Pawe, South Western Ethiopia, where the whole forest flowered and died in 1998 (Kassahun, 2003). Currently, gregarious flowering of bamboos is causing total death of the plants in Bensgangul gumuz region (Demissew Sertse, Personal communication). In addition to seed abortion just after fertilization, short viability of seeds further restricts the availability of seeds as and when required for planting program.

On the other hand, the effectiveness of cuttings to establish themselves for vegetative propagation is very low. Moreover, those conventional propagules of propagation count their ages from their mother plant. This leads to a subsequent death of the newly regenerated plants at the same time with their parent clumps which can cause a catastrophic loss of the plant ecosystem. Rhizomes and offsets can be alternatives for asexual propagation. However, in addition to the wastage of land, planting materials and time to obtain them, they are neither effective in regeneration nor economical for mass

propagation. They also count their ages from their mother plants to flower and die shortly. Besides this, new bamboo plants cannot grow from a clump unless and otherwise the mother plants feed them till they get established (Gielis, 1995).As all in situ clonal materials are at possible verge of loss, *in vitro* culture may help in retaining materials.

Cultivation of bamboo could bring an economic boost for the local farmers as well as commercially for investment. Hence, availability of quality materials is a major input for viable business from this sector. The traditional cultivation system, with the natural succession of the bamboo plant in the forests, is rendering less income that could be affected by genetic variability, unavailability of seeds or planting materials. Bamboo tissue culture could be a way out from problems associated with propagation of the plant using conventional techniques, and to promote solid business from this sector for local, national as well as international market. According to Gielis (1995) and Arshad *et al.* (2005), tissue culture propagated plants have proved to perform better than types of planting resources like cuttings.

Research on the tissue culture of bamboos is fairly recent except for the report by Alexander and Rao (1968) on the culture of bamboo embryos and their germination in to plants *in vitro*. Since then, protocols were optimized for *in vitro* propagation of many bamboo species of the world (Nadgir *et al.*, 1984; Kalaptaru and Mina, 2008). The availability and presence of active meristematic tissue have been the primary considerations for the selection of explants. The explants which were used for callus induction in bamboo are mature embryos, young florets, internodal sections (including the intercalary meristem), leaves, leaf bases, shoot tips and regenerated roots.

In the case of Ethiopia, there was no attempt made to develop a tissue culture protocol for bamboo species so far. There is a huge threat facing on bamboo biodiversity due to the current mass flowering and subsequent death of the species at the same time. This nation also needs to grip the income from the ever growing commercialization of bamboo plantations and marketing of bamboo products. Once the appropriate micropropagation protocol is in place, activities can commence to mass propagate the plant in its ecology to

offset any decline that may occur due to the natural death of large population of bamboo as well as gear inputs of solid business that could satisfy the ever growing national and international markets from this sector. Therefore, the objectives of this research work were:

General Objective

The general objective of the current study was to optimize a protocol for *in vitro* mass propagation of *Oxytenanthera abyssinica*, A. Rich.

Specific Objectives

- To determine the optimum concentration of BAP for *in vitro* initiation of *O. abyssinica,* A. Rich;
- To determine the optimum PGR concentrations for *in vitro* shoot multiplication of *O. abyssinica,* A. Rich;
- To investigate the *in vitro* rooting response of *O.abyssinica,* A. Rich shoots to different IBA concentrations ;
- To evaluate the percentage survival of *in vitro* generated *Oxytenanthera abyssinica*, A. Rich plantlets after acclimatization.

2. LITERATURE REVIEW

2.1. World production and uses of Bamboo

It has been estimated that the combined value of internal and commercial consumption of bamboo in the world is to the tune of US$10 billion, projections are that it will reach between USD 15-billion and USD 20-billion in 2017 (INBAR, 2011)(http://www.inbar.int/index.ASP). China is by far the largest exporter with 46% market share followed by Indonesia (16%) and Vietnam (5%). The biggest importers are European Union (29%), USA (18%) and Japan (10%) (Ramanuja *et al.*, 2008).

In addition to the environmental and biodiversity benefits, bamboo industrial operations, with their superior strength, moisture resistance and pest-proof properties will be useful as building materials, automobile and railway carriage interiors and for packaging. School buildings and tourist places are ideal for using bamboo panel products and flooring materials and furniture. In Asia, bamboo is quite common for bridges, scaffolding and housing and production of boats, boards, doors, attractive furniture etc. Bamboo for power generation and biofuel could be practical and economical in some situations (Ranjan, 2005).

The bamboo products such as rayon, cellulose fibre and pulp plants for export of high quality bamboo clothing , bath robes, towels, baby wear, T-shirts, socks, jackets and suits are being sold in high priced department stores in the west at high prices. In Japan, a bamboo fibre suit is sold at US$ 7,000. It looks like in China alone, there are over a 100 companies involved in bamboo textiles as evidenced by a quick Google search. A sports jacket in Toronto at Harry Rosen is going for C$ 750, given its long history and expertise. Bamboo pulp has other uses besides paper (Ramanuja *et al.*, 2008).

Many specialized items such as musical instruments, activated charcoal, surf boards and pharmaceuticals have small but lucrative markets. Extracts from various parts of the plant have been used for hair and skin ointment, medicine for asthma, eyewash, potions for lovers and poison for rivals. Bamboo ashes are used to polish jewels and manufacture electrical batteries. It has been used in bicycles, dirigibles, windmills, scales, retaining

walls, ropes, cables and filament in the first light bulb. Indeed, bamboo has many applications beyond imagination of lucrative industrial applications (Ramanuja *et al.*, 2008). Last but not the least will be promoting bamboo for carbon sinks. Bamboo plantations could be promoted to become part of a global carbon credit mechanism to benefit the country and attract investments. Bamboo has several advantages over tree species in terms of sustainability and carbon fixing capacity (Jiang, 2007).

2.2. Production constraints of Bamboo

Lack of scientific methods for propagation and cultivation, lack of post-harvest treatment and technology for product development, inadequate trained workforce and inadequate infrastructure for large-scale harvesting in the event of gregarious flowering are the most priority areas that reduce the production (Kigomo, 2007). The intriguing phenomena of gregarious flowering indicate that it is not brought about by external factors but is defined by an internal mechanism of gene activity. Flowering, when bamboo reaches a certain age, and the regular fixed flowering cycles in certain species, indicate an age-related gene activity. At the same time, it does not imply that environmental factors do not have an influence (Gielis, 1995).

In Ethiopia, the gregarious flowering and eventual death of all bamboo trees in a forest is a characteristic that may seriously affect the sustainable supply of raw materials for bamboo-based industries (Kassahun, 2003). In recent years, flowering of bamboo in many places of Ethiopia is resulting in heavy losses of bamboo biodiversity leaving the lives dependent in bamboo in danger. This nation is not effectively utilizing the bamboo resources at this time disposal. Hence, mass propagation of bamboo is pioneer effort to save the biodiversity from jeopardize and acquire income from this sector (Demssew *et al*., 2011).

2.3. Conventional propagation techniques of Bamboo

Bamboo is a critical resource, which has not easily lent itself to modern methods of micropropagation and genetic improvement owing to its long vegetative phase and monocarpic flowering behavior. Conventional breeding is difficult because of the near impossibility of getting two desirable parents to flower simultaneously. Thus, to meet the

raw material demand, the best possible way to manage the bamboo forest is through scientific management. Major limitations to bamboo production have been overcome by propagation methods.

Seeds of bamboo are presumably not available. In fact, only a few people are aware that bamboos produce seeds. They abort soon after flowering and stay viable for short period of time. It may take a century or even more for certain species to produce seeds and the exact period for this to occur is impossible to predict. Due to long flowering intervals, poor seed setting, synchronous flowering and death, short viability, lack of storage methods for recalcitrant seed types, availability of seeds every year for production of planting stock of a selected species is the main bottleneck in establishing bamboo plantations on regular basis from seeds. Since, bamboo is one of the plants that fertilize by out crossing; the resulting seeds are genetically variable too (Demssew *et al*., 2011).

Vegetative propagation methods like rhizomes and offsets are on the other way limited in number, labor intensive for preparation, heavy and difficult to transport, and flower with that of parent clumps. Naturally, the mother plant helps the growth of the newly emerging clumps. Hence, they mostly fail to sustain their life when they are planted independently. Cuttings have low rooting percentage in addition to the constraints listed for the other means of vegetative propagation. Synchronous flowering of vegetative propagated plants with that of parent clumps that result in eventual death of the plants is one of the main pitfalls that enforce us to select seed grown plants than vegitatively propagated plantations (Demssew *et al.*, 2011).

2.4. Tissue culture of Bamboo

Tissue culture is playing a major role in realizing this objective for the production to meet the demand. Tissue culture offers enormous potential in producing large quantities of genetically uniform proliferates of the desired material in a short time frame. However, it is essential that enough care is taken in selection of the initial material, production of the plants, nursery development and field plantation. The *in vitro* methods offer an attractive alternative to conventional methods for the mass propagation of bamboos (Gielis, 1995). Micropropagation of bamboos from mother plants grown from seeds is the far better

approach by no question in supplying enough planting materials for commercialized plantations through mass propagation.

Most reports dealing with bamboo tissue culture have come from Asia, particularly India, China and Thailand. This is possibly due to the fact that nearly three fourth of the total bamboo reserves in the world are located in this region. Although the pioneering report describing regeneration of bamboo plantlets through embryo culture appeared way back in the late 1960s (Alexander and Rao, 1968), extensive *in vitro* studies on bamboos started only about two to three decades ago. After the research on tissue culture of bamboo by Alexander and Rao (1968) on embryo culture of *Dendrocala strictus,* a good start has been made on bamboo tissue culture and a number of laboratories have begun to make progress. But, the majority of the successful achievements are made through somatic embryogenesis and/or regeneration from juvenile seedlings. Consequently, reports on regeneration from mature/field source materials followed by up scaling work for commercial use are limited (Kalpataru and Mina, 2008). A complete protocol for micropropagation of Bambusa bamboos (*Bambusa arundinacea*) was published for the first time in 1982 (Mehta *et al*., 1982).

A selected number of private laboratories have succeeded in mass propagating one to many bamboos, either from seedlings or from mature plants. The quality of young plants derived from tissue culture is generally excellent. The plants are vigorous growers and in many ornamental bamboos, the quality of plants of one year old plants derived from tissue culture is considerably better than of those propagated through conventional methods of propagation. Micropropagated plants are also price competitive with other plants (if one includes quality and other long term factors), while for mass propagation of many bamboos, micro propagation is the only technique. As reported by Gielis (1995), micropropagation via axillary branching is a universal technique but above all, it is the best available technique and will become the standard for mass scale propagation of bamboos.

In East Africa, Research on tissue culture of local bamboo at the Kenya Forestry Research Institute (KEFRI) is ongoing. The Research is focused on *Yushania alpine* and

Oxytenanthera abyssinica. Research and developments on bamboo tissue culture in other countries have shown that this method is a very promising alternative source of bamboo planting stock (Kigomo, 2007).

Propagation using axillary buds (Jimenez *et al.*, 2006) have effectively been used to multiply bamboos *in vitro*. This procedure has been implemented successfully in several bamboo species with high multiplication rates. This method will be of choice for mass scale propagation of bamboos because the regenerated plants are genetically uniform. Since the diversity of bamboos is so vast, it is difficult to present a unique step-by-step protocol for micro propagation of all plants classified within this group. Many scientists have succeeded in developing a common micro propagation protocol for at least 60 temperate and tropical bamboos through axillary branching, but commercial interests have made the procedure elusive for the use among the scientific community (Jimenez and Guevara, 2007).

2.4.1. Selection of mother plant

The lowland bamboo species (*Oxytenanthera abyssinica*, A. Rich) accounts 85% of the bamboo plantation in Ethiopia and is believed to be the most widely adopted species that could grow in soils with poor fertility too. Woldemichael (1980) estimated the lowland bamboo covers about 1,000,000 ha. But according to Luso consult (1997), this figure could be based on unverified assumption. Luso consult (1997) estimates area of lowland bamboo in north western part of Ethiopia between 200,000 and 350,000 ha which makes the estimation of lowland bamboo between 700,000 and 850,000 ha; though settlements and death after flowering may have reduced some of the bamboo areas.

The average annual stem increment of the unmanaged natural bamboo forests of Ethiopia is 8.5-10 tonnes (t) of oven-dry matter per ha. This is a higher production rate than reports from bamboo forests in tropical Asia and elsewhere. It is thus possible to harvest about 3 million tons per year of oven-dry biomass on a sustainable basis from the 1 million hectares of bamboo in Ethiopia; assuming selective felling of culms three or more years of age, this could be used to supply part of the particle board, fiberboard, pulp, furniture, construction and energy requirements of the nation if mass production is

achieved. This species is well adapted to our condition and hence, rescue of our biodiversity must be at our hands.

Appropriate establishment of mother plants is the priority consideration for every successful mass propagation program. As it was discussed earlier, the bamboo plants flower once in their life time like any other grasses. But, what makes more interesting here in mother plant selection unlike the other species is that, bamboo plantlets count their ages from their mother plants unless they are raised from seeds. Hence, explants should be taken from seed grown mother plants.

2.4.2. *In vitro* initiation and multiplication of Bamboo

Cytokinins are the group of plant growth regulators which have the ability to release axillary bud from their dormancy (Thimman, 1934). The proliferation and emergence of axillary shoot is stimulated by incorporating the cytokinin, BAP, in the medium. The BAP followed by Kinetin has been employed in shoot tip cultures (Hu and Wang, 1983). BAP was found to be more effective cytokinin for axillary bud proliferation and multiple shoot production. This has been proved with a number of Bamboo species like *Dendocalanzrs slrictrs* (Banik, 1985), *Bambusa wamin* (Arshad *et al.*, 2005), *Guadua angustifolia* Kunth (Jimenez *et al*., 2005), *Bambusa vulgaris* (Ndiyaye *et al.*, 2006), *Bambusa balcooa* Roxb (Kalpataru and Mina, 2008), *Gigantochloa atroviolaceae* (Bisht *et al.,* 2010) etc. BAP is the most active and the cheapest cytokinin that can be autoclaved. Therefore, it is the one most often used, particularly in commercial micropropagation establishments where cost and ease of handling are major considerations.

Breaking of nodal buds and sprouting of shoots depend on the condition of explants, season of the year and culture conditions. Ramanayake *et al.* (1995) studied *in vitro* bud breaking of two bamboos (*Dendrocalamus giganteus* and *Bambusa vulgaris*) from April, 1994 to April, 1995 and found seasonal effect on bud-breaking. Similar observations were also made by Saxena and Dhawan (1994) on *D. longispathus*.

In the bamboo species, *Guadua angustifolia* Kunth, highest bud sprouting in original nodal explants was observed when 3 mg l^{-1} 6-benzylaminopurine (BAP) was incorporated into the MS culture medium. Production of lateral shoots in *in vitro* growing plants increased

with BAP concentration in culture medium, up to 5 mg l^{-1}, the highest concentration was assessed. After six subcultures, clumps of 8–12 axes were obtained and their division in groups of 3–5 axes allowed multiplication of the plants (Jimenez *et al*., 2006).

On the other hand, in *Melocanna baccifera,* axilliary bud break was observed in nodal segments within 15-20 days, when cultured on MS medium supplemented with BAP. The morphogenic response of explants towards axillary bud proliferation was markedly influenced by the concentration of growth regulator in the medium. Nodal segments cultured on liquid MS medium without plant growth regulator, yielded only 10.33% bud break response. Amongst cytokinin tried, BAP proved its superiority in inducing multiple buds. Maximum bud break response (82.80%) was obtained on liquid MS medium supplemented with 20 µM BAP. However, among kinetin treatments, maximum responding explants (55.40%) were recorded at 25 µM concentration. *In vitro* differentiated shoots were further multiplied on liquid MS medium supplemented with 15µM BAP and 3µM 6-furfuryl amino purine (Kin) at a rate of 2.99 folds, every 4 weeks.

In *Bambusa wamin* (Arshad *et al*., 2005), multiple shoots were induced on Murashige and Skoog medium supplemented with 5.0 mg l^{-1} 6-benzylamino purine (BAP). On the other hand, Ndiyaye *et al. (*2006) reported optimal shoot growth after 16 days cultivation on Murashige and Skoog (MS) medium supplemented with 2 mgl^{-1} of BAP in *Bambusa vulgaris.* Elsewhere, in *Bambusa balcooa* Roxb (Kalpataru and Mina, 2008), multiple shoot formation was observed from excised tender node (12–18 mm in length) containing axillary bud isolated from secondary branches of 1½-yr-old culms, when implanted on Murashige and Skoog (MS) medium containing 1.0 mgl^{-1} BAP).

Bisht *et al.*(2010) reported that, axillary bud break was accomplished in full strength liquid MS medium fortified with 25.0 µM BAP. Axillary shoots produced were multiplied on semi-solid MS medium supplemented with BAP (20µM) + NAA (3.0µM) giving a multiplication rate of 2.39.

According to Sanjay and Sant (1993), culture initiation of *Dendrocalamus longispathus* was strongly influenced by the nature of the explants and the season. *In vitro* multiplication was achieved through forced axillary branching. Single node segments from the young

lateral branches produced multiple shoots on agar-solidified MS medium supplemented with 12 µM benzylaminopurine (BAP) and 3 µM Kinetin. The shoots were multiplied for 15 passages in liquid and thereafter for over 5 passages on semisolid MS + 15 1µM BAP + 1 MM indolebutyric acid (IBA) + 10% coconut water at a rate of 3.2- and 2.8-fold, every 4 week, respectively. The nature of the propagule was a critical factor for shoot multiplication and subsequent rooting.

Nodal segments from field grown culms were used as explants to develop a method of *in vitro* plantlet regeneration in *Bambusa glaucescens (*Willd) through axillary bud proliferation. Shoot multiplication experiments were carried out with different concentrations of benzyl adenine (BAP) and kinetin (Kin), either singly or in combination. A synergistic effect of the two cytokinins was observed and the best interaction giving the highest rate of shoot multiplication (4.00-fold) was obtained for a combination of 5 µM BA and 15 µM Kin. A significant decrease in shoot length was observed up on the addition of BAP to the medium. The longest shoots were obtained on the control (2.76cm). Similarly, shoot length also decreased significantly on Kin supplemented medium and the maximum shoot length was obtained on the control (2.97cm). A significant effect of BAP and Kin interaction was also observed on shoot length. All interactions between BAP and Kin significantly reduced the shoot length. The maximum shoot length (3.45cm) was obtained on the control, which was statistically higher than the shoot length obtained on all other combinations (Fatima and Rana, 2008).

Inter nodal explants of *O. traiancorica* was proliferated in different concentrations of BAP. The maximum multiplication rate was observed in 4 mgl^{-1} BAP, which was same as with the results obtained in the experiments in *Dendrocalamrs strictus.* However, the number of multiple shoot recorded in *O. lravancorica* was higher than that reported in *Hanibzrsa vulgaris* by Nadgir *et al.* (1984). The BAP at higher concentration ($8mgl^{-1}$) induced increase in the number of multiple shoots in *O. travancorica*. But, the shoot induced was pale, weak and short. High cytokinin concentration has suppressive effect on shoot elongation in woody plants. The high concentration of cytokinin may cause toxic effect on cultures (Davies, 1988). On the other hand, greatest shoot length was obtained when the medium was supplemented with 0.5 µM BAP (Gomez and Segura, 1995).

Kinetin promotes the multiple shoot production. Kinetin has shown considerable role in the multiple shoot induction in *Banbusa vulgaris* by Nadgir *et al*. (1984). The effect of Kinetin was more noticed in the stage II, multiplication medium. Significant positive effects of BAP and Kin interactions on the multiplication of shoots were noted. High rates of shoot multiplication in the presence of both BAP and Kin as compared to BAP or Kin alone suggest as synergistic interaction of the two chemicals (Fatima and Rana, 2008). A synergistic effect of 15 µM BAP and15 µM Kn combination was also reported by Shirin *et al.* (2003), which resulted in a high rate of shoot multiplication in *Bambusa vulgaris* from axillary buds of mature culms. Of the various multiplication media that were used , the best results were obtained on MS medium supplemented with BAP ($2.5x10^{-5}$M) + Kin ($5x10^{-6}$M). On this medium, the shoots multiplied at a rate of 2.4 fold every 6 weeks (Saxena, 1993).

In *Bambusa tulda*, multiple shoot formation was achieved within 10-12 days on MS medium containing BAP ($l.5x10^{-5}$M) and Kn ($4x10^{-6}$M). These shoots were multiplied for two passages at a rate of 4 fold every 3 weeks. Thereafter, the multiplication rate declined drastically and by the end of the 5th passage, all the shoots became dormant. These shoots failed to rejuvenate and finally died. The study suggests a strong maternal influence, which could not be overcome by manipulation of phytohormones and other growth nutrients present in the culture medium or change in physical conditions (Saxena, 1990).

Different types of auxins and cytokinins were used for their effect on multiple shoot induction. A reason of the positive effect of auxin at low concentration in the culture media is that it nullifies the effect of higher cytokinin (Hu and Wang, 1983). A cytokinin /auxin combination has earlier proved to be efficient for *in vitro* shoot proliferation in *Pseudoxytenanthera stocksii* (Sanjaya *et al*., 2005), *Bambusa nutans* (Yadav *et al.*, 2008) and *Oxytenanthera abyssinica* (Mohammed and Diab, 2008). Contrastingly, in some studies on micropropagation of bamboos, either BAP alone or a combination of BAP and Kin was found to give best results of shoot multiplication *in vitro* (Nadgir *et al*.,1984; Ramanayke and Yakandwala , 1997; Das and Pal, 2005; Sanjay *et al.*, 2005; Kapoor and Rao, 2006).

The auxins like IAA were used for the multiple shoot inductions. The 1AA (l mgl^{-1}) was found to have no effect on axillary bud proliferation and multiple shoot induction. Further increase in the IAA up to 5 mgl^{-1} produced slight proliferation of the axillary bud . The same effect was also observed in O.*lravancorica*. The earlier micro propagation on bamboo reviewed showed that IAA had no effective role in axillary bud proliferation and multiple shoot induction (Prutpongse and Gavinletvatana, 1992).

2.4.3. *In vitro* rooting and acclimatization of Bamboo

The ability of plant tissues to form adventitious roots depends on the interaction of many different endogenous and exogenous factors. Skoog and Miller (1957) reported that the shoot/root formation is generally dependent on the cytokinin/auxin ratio in the nutrient medium. Jones (1978) demonstrated that the cytokinin inhibit rooting and also prevent the root growth. Therefore, it is better to culture the shoots on a PGR free medium before they are transferred to a rooting medium.

In relation to the micropropagation of adult plants of bamboo, the success has been rather restricted. Nadgir *et al.* (1984) made a breakthrough in multiplying shoots derived from nodal explants of adult Bambusa bamboos, *B. vulgaris* and *Dendrocalamus strictus*. However, rooting occurred only in *D. strictus* and that too, at a very low frequency (maximum of 20%). Chaturvedi *et al.* (1993) failed to multiply *D. strictus* shoots but succeeded in inducing 30% rooting along with regeneration of axillary shoots when nodal segments were cultured upside down on a complex medium containing phloroglucinol.

In other studies, rooting percentages for adult bamboos ranged from very low percentages of 10% for *Bambusa vulgaris* to 73% for adult *Dendrocalamus longispathus* (Saxena and Dhawan, 1994). A rooting percentage of 77% was obtained for adult *Dendrocalamus giganteus* in 3 or 4 weeks (Ramanayake and Yakandawala, 1997). Nonetheless, low rooting frequencies are the major bottleneck to developing commercially viable protocols.

The rooting media used for *O. ravancorica* shoots were similar to those used in many other bamboos species: i.e. lowered mineral salts and higher auxin (Nadgir *et al.* 1984). Experiments revealed that IBA, NAA alone or combination was found effective in root induction. The auxin concentration varies from 1-5 µM. The presence of activated

charcoal ($8mgl^{-1}$) in the media was found effective in rooting of other bamboo species like *Banihuta vtrlgaris* (Nadgir *et al.*,1984). The effective mechanism of activated charcoal in the tissue culture media for root induction was not clearly identified (George and Sherrington, 1984). Studies on *B. vtrlgaris* (Prutpongse and Gavinlertvatana, 1992) reported that NAA and 1BA were efficient for *in vitro* rooting of shoots. Addition of IBA and NAA together was found to improve the root induction. The combination of NAA with IBA induced maximum roots.

Corresponding to some of the above works, 73% of the shoots rooted on a modified MS medium (major salts reduced to half strength) containing 1µM indoleacetic acid, 1 µM IBA and 68 µM coumarin and through a simple *in vitro* hardening step, more than 85% of the tissue culture-raised plants were successfully transferred to soil (Sanjay and Saint, 2003). The MS medium supplemented with 25 µM IBA was most suitable for rooting of shoots. Hardening and acclimatization was successful and plantlets are growing normally in soil (Fatima and Rana, 2006). On the other hand, *in vitro* shoots were rooted on full strength MS medium supplemented with varying concentrations of auxins. Optimal rooting was achieved on medium supplemented with 35.0 µM IBA. Regenerated plantlets were successfully hardened and acclimatized under net house conditions with over 80% survival (Bisht *et al.*, 2010).

Although, a variety of auxins (IAA, IBA and NAA) and other growth regulators such as phloroglucinol, coumarin, activated charcoal and boric acid were tested to induce rooting, only a maximum of 10% rooting was achieved when shoots were cultured on MS medium supplemented with IAA, IBA and coumarin (100 mg/l each) for two days and then transferred to a hormone-free MS medium. Attempts to improve rooting frequency by altering physical environment also proved futile. According to Saxena (1990), low rooting frequency was the major bottleneck in the establishment of a commercially viable protocol of *B. vulgaris*.

With these more than 45 genera of bamboo species, it is neither possible to deduce from one protocol to another protocol nor specious to predict through pilot studies. Many works have still ended up with null or small (up to 10% rooting efficiency) rooting in some

species. In contrast, the misery goes divulged as there are some plants that bear indigenous *in vitro* roots easily.

Despite all the fuzz, some plants easily form roots *in vitro*. This can be an advantage or disadvantage. Because, *in vitro* roots are not always functional and they are also likely to be damaged during planting out. These roots usually die and new roots have to be formed once the plant is established. In such a case allowing the plants to root outside via *ex vitro* rooting in the glasshouse would be better. Many tissue culture laboratories commonly use *ex vitro* rooting. This reduces the costs and time taken for production of rooted plantlets. Some crops root much easier *ex vitro* than *in vitro*, this is especially true for woody plants for example pistachio rootstock (Sanette, 2001).

In another case, *in vitro* rooting can be further forced by subjecting the shoots to extremely high auxin levels. Hence, optimal rate of rooting (45.83%) was observed in *B.vulgaris* in MS medium supplemented with 20mg/l IBA. The high concentration of IBA (20 mg/l) added to the MS medium could be explained by the lack or the feeble synthesis of endogenous auxin by the explants (Ndiyaye *et al*., 2006). However, according to El Nour *et al.* (1991), this higher concentration of rooting (IBA) hormone did not improve rooting significantly in bamboo tissue culture. Availability of endogenous hormone suppresses exogenous effect (Andrew and Bonnie, 2004)

3. MATERIALS AND METHODS

3.1. Experimental materials

All the laboratory activities and experiments were conducted at the National Agricultural Biotechnology Laboratory under the Ethiopian Institute of Agricultural Research, Holleta Agricultural Research Center. Mother plants of *Oxytenanthera abyssinica* were grown from seeds and maintained in a glass house for a year with regular watering every three days. The seeds were obtained from Komosha area, Benshangul Gumuz Region. *Oxytenanthera abyssinica*, the major bamboo species that covers 85% of bamboo populations, thrives in very poor and shallow soil which is unsuitable for most cereal crops. The species is very drought resistant, sustains itself with minimal rainfall and has a very economical water uptake. This species is non invasive, clumping (symbodial) bamboo, with short slow-spreading rhizomes arising close to the mother culms. This species also grows in many parts of the SNNP, Gambella, Oromia, Amhara and Tigray regions .

The plants were raised in plastic pots containing soil, compost and sand in the ratio of 2:1:1, respectively and allowed to grow in the glasshouse. After a year of growth, mother plants that developed active buds in their nodes were used as source of explants (Appendix Figure 1). All the basal media components, hormones and tissue culture lab set ups were used in the national plant biotechnology laboratory.

3.2. Media Preparation

Murashige and Skoog's medium (1962), containing 3 % sucrose and 0.5% agar as a solidifying agent, was used as a basal culture medium throughout the experiments. Full strength stock solutions of macronutrients, micronutrients and vitamins and other organic supplements were prepared in six separate volumes of stock solutions (Appendix Table 1). To do so, appropriate amount of each nutrient was weighed in grams per liter and dissolved in double distilled water consecutively in such a way that the next nutrient was added after the first one was completely dissolved. After all the components were completely dissolved using magnetic stirrer, the solution was poured into plastic bottles and stored at $+4^0$C for maximum of four weeks until used.

Plant growth regulators (PGRs) were prepared in 1mg/ml concentration. The PGRs used for the study were cytokinins: 6- benzyl amimopurine (BAP) and Kinetin, the auxins: indol-3- butyric acid (IBA), Naphtalene acetic acid(NAA) and Indole acetic acid (IAA) and gibberellin: gibberrellic acid (GA_3). The powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N HCl for cytokinines, 1N NaOH for auxins and 99% ethanol for gibberellin (Sanette, 2001). Upon complete dissolution, the solution of each PGR was poured into labeled 50 ml plastic bottles and filled with double distilled water to the required volume. Then, it was gently stirred and stored at a temperature of $+4^0$ C.

In the first round of experiments, the culture media for shoot initiation and multiplication contained full strength of MS basal medium with or without (for control) PGRs. In the rooting experiments, different strengths of MS basal medium were used with or without different concentrations of IBA, NAA or IAA. After adding the respective PGRs, the pH of all media was adjusted to 5.7 by using 1N HCl and/or NaOH.

After addition of 0.5% agar, the solutions were gently mixed and boiled on a stove until the agar gets melted. Then, 50 ml of the prepared medium was dispensed into a culture vessel. The culture vessels were covered with caps immediately after dispensing and the medium autoclaved by steam sterilization at a temperature of 121^0c and 105 KPa pressure for 20 minutes. Immediately after autoclaving, the medium was taken and kept in laminar air flow cabinet bench until it is solidified and cooled. The solidified media were kept in a shelf for about a week in order to see some indications of microbial contamination before culturing explants for initiation or shoots for multiplication and rooting.

3.3. Explant Preparation

Nodal segments from the 2^{nd} and 3^{rd} nodes with a length of 2.0 -2.5 cm were used as explants. After cutting the explants, they were transferred to the preparation room of the laboratory in a vessel containing distilled water. After preliminary washing, the culm sheaths were removed by a blade.

Prepared explants were washed under running tap water and with the addition of detergents three times for 10 minutes and then treated with 0.3% Kocide (fungicide solution) for 20

minutes. Later, they were washed by 2% sodium hypochlorite (NaClO) solution containing two drops of tween 20 solution for 10 minutes inside a laminar air flow hood (Appendix Figure 2 A). Then, explants were surface sterilized with 70% ethanol for one minute. After rinsing with distilled water for five minutes, the explants were washed again by 1% sodium hypochlorite solution containing two drops of tween 20 solutions for 10 minutes. Finally, they were washed with double distilled deionized water three times. Subsequently both ends were trimmed (Appendix Figure 2 B) and segments cultured on Murashige and Skoog's (1962) medium (50ml) with plant growth substances by placing them vertically (75^0) in a conical culture vessel (250 ml) (Appendix Figure 2 C).

3.4. Culture conditions

After culture of explants or sub culturing of shoots, the culture vessels were properly sealed with parafilm, labeled and transferred in to the growth room. The cultures were kept in the shelf in random and they were maintained at 2500 µmol/m^2 s^{-1} intensity of light (the fluorescent lights were 28 cm away from the top of culture vessels) for 18/6 hours photo period (cool white fluorescent light) and a temperature of 25 ± 2°C. These culture conditions were maintained thoughout initiation, multiplication and rooting stages of all the cultures in the growth room.

3.5. Effects of plant growth regulators on initiation, shoot multiplication and rooting

3.5.1. The effect of BAP on in vitro *initiation of* O. abyssinica *(A. Rich)*

The MS medium was separately supplemented with four levels of BAP (0, 2.5,5 and 10 mg/l) replicated three times only for preliminary trials. After this pilot study, seven levels of BAP (0, 1.5, 2, 2.5, 3, 3.5 and 5 mg/l) were used for final treatment levels for the actual initiation experiment. There were five explants per culture vessel, and each vessel was considered as a replication. The experiment had 6 replications; hence, there were 30 observational units per treatment. Data for days to initiation were recorded every day at the same hour starting from the first day of culture. After 21 days of incubation, number of shoots initiated and shoot length (cm) were recorded. The productions of multiple shoots and nodes from a single explant in the initiation stage were also taken in to account.

3.5.2. The effect of different hormone concentrations on *in vitro* shoot multiplication of *O. abyssinica* (A. Rich)

Two centimeter long, young and healthy micro-shoots obtained from initiation stage were used for the multiplication experiment. The micro-shoots were cut and transferred into each fresh culture vessels under aseptic condition in a laminar flow hood. For preliminary studies, the response of nine levels of BAP (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 5 and 10 mgl^{-1}) and seven levels of Kinetin (0.5, 1, 2, 2.5, 3, 3.5 and 4 mgl^{-1}) combinations were evaluated. The actual experiment was laid out in the combinations of five levels of BAP (0, 1, 1.5, 2.5 and 4 mg/l) and four levels of Kinetin (0, 0.5, 1.5 and 2.5 mgl^{-1}). Three micro-shoots were cultured in a single culture vessel and each vessel was considered as a replication. The experiment had 10 replications; hence, there were 30 observational units per treatment. The experimental shoots were sub-cultured every three weeks for three times by using the best four treatment combinations that gave higher multiplication rate in the 1^{st} stage of growth. All the three steps of sub-culture were made by transferring the newly multiplied micro-shoots to fresh medium of the same composition as the previous one. After 21 days of culture growth in each stage, number of nodes per shoot, number of buds per explant and shoot length were recorded.

3.5.3. The effect of IBA on *in vitro* rooting of *O. abyssinica* (A. Rich)

The multiplied shoots were maintained on PGR free MS medium for two weeks before being transferred to the rooting media. Before culturing, the shoots were cut in a 'V- shape' form to increase the surface area of wounding of the cut end of the shoot which in turn increases rooting efficiency. Simply for preliminary studies, the shoots (3cm long) were transferred to full, ¾ , ½ and ¼ strength MS medium augmented with thirteen different concentrations of IBA (0,0.5,1,2,4,8,10,12,15, 20, 35, 45 and 50 mg/l), six levels of NAA(0,0.25,1,2.5,5,10 and 15 mg/l) and four levels of IAA(0, 0.5,1,2.5,5) in the presence or absence of charcoal. The rooting efficiency in PGR free medium was further tested by addition of phloroglucinol and 60 g/l sucrose. The MS media components that could be responsible for rooting like boric acid, calcium and iodine were also tested.

After this pilot study, three levels of IBA (0, 0.25, and 25) in full strength medium free of activated charcoal were used for the final experiment of *in vitro* rooting. There were three

shoots per culture vessel and each vessel was replicated ten times that constitute 30 observational units per treatment. After 30 days of culture growth, number of main roots per shoot, length of roots (cm) and percentage of rooting were recorded.

The experiment of *in vitro* rooting of *O. abyssinica* was also tested in another different step; exposing to a high concentration (50 mg/l IBA) for some days and then transferring to a hormone-free medium. On the other hand, shoots were kept in PGR free medium for a month and then direct acclimatization through *ex vitro* rooting of the respective plantlets was evaluated.

3.5.4. Acclimatization of plantlets

In vitro rooted plantlets were taken out of the culture flasks and thoroughly washed with water to remove all traces of the medium including agar in order to avoid carry over effect of the rooting medium. Both the *in vitro* rooted and shoots for *ex vitro* rooting were labeled and planted in plastic pot containing a mixture of sieved sand, silt and well decomposed farm yard manure in a 1:1:1 ratio in a tray. The transplanted plantlets were kept under shade in a green house. Humidity was maintained by covering the trays with polyethylene sheets on appropriate supports for 10 days. Initially, there were 30 plantlets for each observation of *in vitro* rooted shoots and shoots for *ex vitro* rooting. The acclimatized plants were further transferred to plastic pots containing soil, compost and sand in the ratio of 2:1:1, respectively and allowed to grow in the glasshouse for further hardening. After three weeks, numbers of survived plantlets were recorded for each observation from *in vitro* and *ex vitro* rooting.

3.7. Experimental design and data analysis

Completely randomized design (CRD) was used for the initiation and rooting experiments. However, the multiplication experiment was laid in factorial combinations of treatments with CRD. Percentage and count data were normalized by logarithmic transformations. The data analysis was made by SAS version 9.0 and Genstat 12th edition. Mean separation was done by Least Significance Difference (LSD) test at 0.05 probability level and standard deviation from the mean of each observation were calculated.

4. RESULTS AND DISCUSSION

4.1. The effect of BAP on *in vitro* initiation of *O.abyssinica* (A. Rich)

ANOVA showed that, different levels of BAP had very high significant effects (P<0.0001) on days to initiation and percentage of initiated explants (Appendix Table 2).

Culturing of explants in the MS medium supplemented with 2.5mgl^{-1} BAP (Figure 1 C) resulted in the shortest days to initiation (4.07 days); which is significantly different from BAP levels of 1.5 (4.42 days), 2.0 (4.40 days) and 3.5 (4.38 days)(Table1). Higher concentration of BAP resulted in late sprouting of the cultures. This could be due to the inhibitive effect of higher hormone concentration in addition to the inherently available indigenous hormone in the explant before inoculation. Successful initiation of explants on PGR free medium (Figure 1 A) is indication of the availability of endogenous hormone. This result was consistent with the works of Bisht *et al.* (2010) and Jimenez *et al*. (2006) in another bamboo species. Other authors also recommended different level of hormone and delayed days to culture, which took 16 days (Ndiaye *et al.*, 2006) and 15 to 20 days (Abhinav *et al.*, 2009). This difference might have arisen due to genotypic difference, which is an established fact, as reported in Prutpongse and Gavinletvatana (1992).

Table 1. Mean number of days to initiation and percentage of initiated explants in MS medium supplemented with different BAP concentrations, mean days are indicated ± SD, means for regeneration percentage are indicated by transformed data ± SD (actual mean).

BAP(mg/l)	Days to initiation	% initiated explants
0	5.01^{d} ±0.18	96.2^{a} ±8.08 (96.7%)
1.5	4.42^{b} ±0.13	96.2^{a} ±8.08 (96.7%)
2	4.40^{b} ±0.13	99.5^{a} ±0.00 (100%)
2.5	4.07^{a} ±0.10	99.5^{a} ±0.00 (100%)
3	4.85^{c} ±0.23	89.6^{ab}±10.26 (90%)
3.5	4.38^{b} ± 0.13	92.9^{a} ±24.26 (93.3%)
5	6.31^{e} ± 0.32	73.1^{b} ±23.98 (73.3%)
Lsd(0.05)	0.21	16.57
CV	3.79%	15.3%

Means having the same superscript letters in the same column are not significantly different at 5% probability level

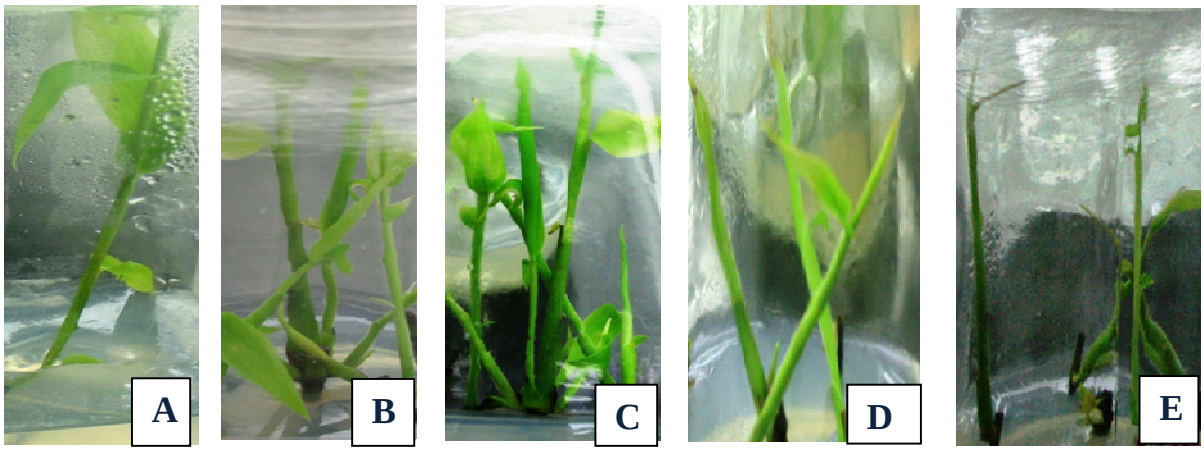

Figure 1. Response of *O.abyssinica* nodal explants to different levels of BAP in the 21th day from inoculation in the MS medium: A) PGR free. B, C, D and E represent MS+2 mgl^{-1}, MS+2.5mgl^{-1}, MS+3mgl^{-1} and MS+5mgl^{-1} respectively.

After 5 days of initiation, 100% of the explants survived and gave effective micro-leaves in the MS medium supplemented with 2 and 2.5mgl^{-1} BAP. However, the PGR free (96.7%), MS+1.5mgl^{-1} BAP (96.7%) and MS+3.5mgl^{-1} BAP (93.3%) showed statistically insignificant results with the treatments that showed in absolute sprouting of all the explants cultured in the medium. The exception was higher BAP concentration (5mgl^{-1}) that showed drastically lower initiation percentage of explants than the others. These results were better than the outcomes of Abhinav *et al.* (2009), which had shown a higher (82.80%) and lower (10.33%) regeneration percentages in *Melocanna baccifera*.

Preliminary observations of multiplication during initiation

ANOVA has shown that, BAP concentration has highly significant effect ($P<0.0001$) on the number of shoots per explant, number of nodes per shoot and shoot length of the cultures during initiation (Appendix Table 2).

Explants cultured on MS medium supplemented with 2.5 mgl^{-1} BAP had shown best number of multiple shoot (2.3) production from a single bud cultured in initiation medium. This medium also showed rapid initiation with relatively tall and thick plantlets. Getting at least two usable shoots from a single explant in the initiation stage means that, the efficiency of micropropagation will be doubled in the available mean time. On the other hand, addition of 3 mgl^{-1}, 3.5 mgl^{-1} and 2 mgl^{-1} to the MS medium has shown satisfactory

sign of production of multiple shoots (1.45, 1.39 and 1.27 shoots/explant, respectively) from a single explant (Table 2).

These results are slightly consistent in terms of multiple shoot production with the works of various authors (Arshad *et al*., 2005; Kalaptaru and mina, 2008; Bisht *et al*., 2010) on *Bambusa wamin* , *Bambusa balcooa* and *Gigantochloa atroviolaceae* , respectively in multiplication stage. However, the results in this experiment are obtained in the initiation experiment and we had separate step of multiplication with outstanding multiplication results.

In terms of multiple nodes per explant, the PGR free medium developed the tallest shoots that gave rise to more nodes (2.87) that can be used for the next step of multiplication. Similar to the days to initiation, higher BAP concentrations were the least concentration for number of nodes per explant. However, the efficiency of these nodal explants for further multiplication up on sub culture was very low. It was difficult to get active buds from these *in vitro* maintained shoots (Data not presented). Therefore, it was found better to use multiple shoots per explant as the main means of multiplication throughout the experiments.

Table 2. Mean number of shoots per explant, nodes per shoot and shoot length in the MS supplemented with different levels of BAP in the initiation medium, means are indicated as ± SD

BAP(mg/l)	Number of shoots/explant	Number of nodes/shoot	Shoot height (cm)
0	1.00^{e}±0.00	2.87^{a}±0.10	6.03^{a} ±0.15
1.5	1.24^{d}±0.08	2.21^{b}±0.13	4.55^{b} ±0.15
2	1.27^{cd}±0.10	1.87^{c}±0.10	4.20^{c} ±0.00
2.5	2.30^{a}±0.11	1.83^{c}±0.15	4.00^{c} ±0.00
3	1.45^{b}±0.15	1.75^{cd}±0.18	3.70^{d} ±0.21
3.5	1.39^{bc}±0.15	1.67^{d}±0.15	3.22^{e} ±0.20
5	1.11^{e}±0.12	1.00^{e}±0.00	3.50^{d} ±0.29
Lsd(0.05)	0.13	0.15	0.20
CV	8.08%	6.77%	4.18%

Means having the same superscript letters in the same column are not significantly different at 5% probability level

Like the node production, explants on plant growth regulator free medium showed significantly higher shoot length (6.03 cm) than any other treatment. The initiated shoots

were thin and long. Addition of BAP to the MS medium resulted in a decrease of shoot length. The shoots tend to become thicker and many shoots per explant are obtained due to the effect of shooting hormone. MS medium supplemented with 1.5 mgl^{-1}, 2 mgl^{-1} and 2.5mgl^{-1} were the next better treatments that resulted in better shoot lengths of 4.55, 4.20 and 4.00 cm, respectively.

Incorporation of multiplication data from initiation culture was found to be supportive for further mass production in the next steps. Because treatments showing advanced proliferation and growth in terms of either multiple buds or nodes were recognized as potential observations for successful multiplication in mass propagation.

Generally, MS media supplemented with increasing BAP concentration showed quicker days to initiation, increase in percentage of regeneration and the number of multiple shoots per explant up to 2.5 mgl^{-1}. However, when the BAP concentration goes beyond that, these parameters showed a decrease in performance. On the other hand, higher BAP was found to be inversely related to the shoot length and number of nodes. Therefore, initiation of explants in the MS medium supplemented with 2.5 mgl^{-1} was found to be the best in terms of quality and profitability wise.

Experiment 2. The effect of different hormone concentrations on *in vitro* shoot multiplication of *O. abyssinica* (A. Rich).

In the 1st stage of multiplication, ANOVA has shown that BAP and Kinetin concentrations as well as their interactions has highly significant effect ($P<0.001$) on number of shoots per explant, number of nodes per shoot and shoot length (Appendix Table 3).

Treatment of shoots with 2.5 mgl^{-1} BAP + 1.5 mgl^{-1} Kin (Figure 2 B) resulted in the highest mean shoot proliferation (5.94 shoots per explant) followed by 1.5m gl^{-1} BAP + 2.5 mgl^{-1} Kin, which gave rise to mean shoot number of 4.97. The other treatment combinations, 2.5 mgl^{-1} BAP+ 2.5 mgl^{-1}Kin and 1.5 mgl^{-1} BAP+ 1.5mgl^{-1} Kin (Figure 2 D) also gave reasonable number of multiple shoots per explant, which produced 4.67 and 4.39, respectively. These treatments gave rise to significantly higher number of shoots per explant than the other treatment combinations in the first culture of multiplication prior to sub-culture (Table 3).

Treatment combinations of higher and lower than 2.5 mg/l BAP and 1.5 mg/l Kin resulted in decreasing level of shoot multiplication, the least of which (1.06 shoots per explant) was obtained on treatment combinations of $1mgl^{-1}$ BAP and 0 Kin. There was no shoot multiplication on the control treatment (Table 3). The results obtained in this experiment showed outstanding rate of multiplication compared to previous experiments on *Bambusa tulda* (Saxena, 1990) and *Bambusa glaucescens* (Fatima and Rana, 2006), which gave 4 - fold multiplication. The multiplication rates on other reviewed reports on *Dendrocalamus longispathus* (Sanjay and Sant, 1992), *Melocanna baccifera* (Abhinav *et al.* 2009) and *Gigantochloa atroviolaceae* (Bisht *et al.*, 2010) were in the ranges of 2 to 3 shoots per explant.

Table 3. Mean number of shoots per explant, number of nodes per shoot and shoot length in MS medium supplemented with BAP and Kinetin in the 1st stage of multiplication, means are indicated as ± SD

BAP (mg/l)	KIN (mg/l)	No. of Shoots / Explant	No. of nodes /shoot	Shoot length(cm)
0	0	1.00^{l}±0.00	3.35^{a}±0.21	7.01^{a}±0.19
	0.5	1.38^{k}±0.17	2.75^{c}±0.25	5.50^{c}±0.19
	1.5	1.76^{j}±0.13	2.12^{f}±0.15	5.06^{d}±0.26
	2.5	2.00^{i}±0.00	1.94^{hi}±0.13	4.86^{de}±0.22
1	0	1.06^{l}±0.13	2.88^{bc}±0.15	5.47^{c}±0.23
	0.5	2.15^{i}±0.16	3.00^{b}±0.20	5.55^{c}±0.20
	1.5	2.88^{g}±0.25	2.00^{fghi}±0.00	5.06^{d}±0.37
	2.5	3.38^{e}±0.17	1.97^{ghi}±0.17	4.96^{d}±0.18
1.5	0	1.52^{k}±0.30	2.58^{d}±0.19	5.56^{c}±0.38
	0.5	3.19^{f}±0.23	2.13^{f}±0.24	5.51^{c}±0.11
	1.5	4.39^{d}±0.23	2.32^{e}±0.23	6.06^{b}±0.24
	2.5	4.97^{b}±0.26	1.97^{ghi}±0.09	4.92^{d}±0.24
2.5	0	2.42^{h}±0.19	2.06^{fgh}±0.13	4.64^{ef}±0.21
	0.5	3.50^{e}±0.21	2.06^{fgh}±0.13	5.64^{c}±0.21
	1.5	5.94^{a}±0.24	2.46^{de}±0.21	5.55^{c}±0.20
	2.5	4.67^{c}±0.29	2.09^{fg}±0.14	4.85^{de}±0.16
4	0	1.49^{k}±0.26	1.42^{j}±0.19	3.95^{g}±0.53
	0.5	2.94^{g}±0.19	2.03^{fghi}±0.09	4.44^{f}±0.16
	1.5	2.78^{g}±0.23	2.03^{fghi}±0.09	3.74^{g}±0.20
	2.5	2.09^{i}±0.14	1.91^{i}±0.14	2.42^{h}±0.71
Lsd(0.05)		0.18	0.15	0.26
CV		7.4%	7.5%	5.8%

Means having the same superscript letters in the same column are not significantly different at 5% probability level

In the case of number of nodes per explant and shoot length, the control treatment (PGR free medium) was the best that resulted in 7.01 cm length of shoot and 3.35 nodes (Figure 2 A). The general trend in the result of number of nodes in a shoot was that as the concentration of BAP and Kin increased, production of nodes decreased. The same was true for shoot length. The exceptions were at combinations of 1.5 mg^{-1} BAP + 1.5 mg^{-1} Kin and 1mg^{-1} BAP + 0.5mg^{-1}Kin that had second best shoot length (6.06cm) and number of nodes (3.00) respectively, which was statistically better than zero BAP and 0.5mg/l Kin (Table 3). The shoot length results were better than those reported by Fatima and Rana (2006) and Gomez and Segura (1995).

As plant growth hormones are expensive, the natural thing would be going for the lowest concentration of hormones without compromising the results; hence, the control treatment (Figure 2 A) can be the one to choose as the best and economical treatment if one considers multiple nodes per shoot as a means of multiplication for the next steps. But production of multiple shoots per explant gave best performance for successful *in vitro* multiplication as the shoots are already established and they can easily proliferate in the next sub culture stage. We can also get almost double amount of multiple shoots than the nodes as revealed in this experiment. Therefore, addition of 2.5 mg^{-1} BAP and 1.5 mg^{-1} Kin in the first stage of multiplication is the best approach for mass propagation of bamboo (*O. abyssinica*).

On the other hand, ANOVA has shown that multiplication stage, BAP and Kinetin and all of their interactions (except BAP*KIN for nodes per shoot), showed significant statistical difference for number of shoots per explant, nodes per shoot and shoot length (Appendix Table 4).

In agreement with the previous observation in multiplication before sub culture (1st stage of growth), the MS medium supplemented with 2.5mgl^{-1}BAP and 1.5mgl^{-1} Kin (Figure 2 B) resulted in the highest number of shoots per explant (5.84) at the 2nd stage of growth. However, it showed insignificant difference with the MS medium supplemented with 1.5mgl^{-1}BAP and 1.5mgl^{-1} Kin in the 3rd and 4th (Figure 2 E) stages of growth with 5.68 and 5.63 multiple shoots respectively. This could be due to the accumulation of these hormones in the successive steps of sub culture.

The multiplication rate of the same treatments in successive stages was decreasing except in the case of the medium supplemented with 1.5mgl^{-1} BAP and 1.5 mgl^{-1} Kin, which showed increasing responses of multiplication throughout all stages. The decreasing multiplication results are in accordance with the outcomes of Saxena (1990) in *Bambusa tulda*, where the multiplication rate declined drastically and by the end of the 5th passage, all the shoots became dormant.

Up to the 2nd step of sub culturing (3rd stage of multiplication), addition of 1.5mgl^{-1} BAP and 1.5mgl^{-1} Kin showed in increasing number of shoots than at every successive stage (Table 4). It produced 4.39, 4.9 and 5.68 shoots per explant in the 1st, 2nd and 3rd stages of multiplication respectively. The mean number of shoots in the 4th stage of multiplication (5.64) was statistically non significant from the 3rd stage(Figure 2 E) multiplication medium supplemented with the same hormone concentration augmented with 1.5mgl^{-1} BAP and 1.5mgl^{-1} Kin. However, it also showed non significant difference with the 2nd and 3rd stages of growth augmented with 2.5mgl^{-1}BAP and 1.5mgl^{-1} Kin.

However, the MS medium supplemented with 2.5mgl^{-1} BAP and 1.5mgl^{-1} showed decreasing number of shoots (Table 4) throughout the sub culture process (Figure 2 C). Of course, this hormone combination produced the best number of multiple shoots in the 1st stage of multiplication. Therefore, there should be a shift of using 1.5mgl^{-1}BAP and 1.5mgl^{-1} Kin in the 3rd and 4th stages of multiplication after the 1st and 2nd stages of growth supplemented with 2.5mgl^{-1}BAP and 1.5mgl^{-1} Kin.

At the same time in each sub culture stage, the MS medium supplemented with 1.5mgl^{-1}BAP and 1.5mgl^{-1} Kin also showed higher number of nodes per shoot and shoot length in except in the medium supplemented with 2.5mgl^{-1} BAP and 1.5mgl^{-1} Kin at the 1st stage of multiplication, which showed statistically insignificant result (2.46 nodes).

Table 4. Mean number of shoots per explant, number of nodes per shoot and shoot length in MS medium supplemented with BAP and Kinetin at different culture stages, means are indicated as ± SD

Stage	BAP (mg/l)	KIN (mg/l)	No. of Shoots / Explant	No. of nodes /shoot	Shoot length(Cm)
1st	1.5	1.5	4.39^{gh}±0.23	2.32^{cd}±0.23	6.06^{b}±0.24
		2.5	4.97^{d}±0.26	1.97^{fg}±0.09	4.92^{f}±0.24
	2.5	1.5	5.94^{a}±0.24	2.46^{bc}±0.21	5.55^{cd}±0.20
		2.5	4.67^{ef}±0.29	2.09^{ef}±0.14	4.85^{f}±0.16
2nd	1 .5	1.5	4.90^{de}±0.31	2.46^{bc}±0.21	6.07^{b}±0.28
		2.5	4.74^{def}±0.31	2.00^{fg}±0.00	5.43^{d}±0.26
	2.5	1.5	5.84^{ab}±0.31	2.18^{de}±0.15	5.63^{c}±0.20
		2.5	4.53^{fg}±0.26	1.90^{g}±0.23	4.75^{fg}±0.26
3rd	1.5	1.5	5.68^{b}±0.23	2.71^{a}±0.26	6.43^{a}±0.29
		2.5	4.60^{fg}±0.38	2.00^{fg}±0.14	4.84^{fg}±0.22
	2.5	1.5	5.68^{b}±0.23	2.21^{de}±0.15	5.37^{ed}±0.14
		2.5	4.09^{i}±0.20	1.91^{g}±0.15	4.65^{gh}±0.25
4th	1.5	1.5	5.64^{bc}±0.26	2.50^{b}±0.21	5.56^{cd}±0.24
		2.5	4.26^{hi}±0.27	1.83^{g}±0.29	4.51^{hi}±0.18
	2.5	1.5	5.43^{c}±0.25	2.33^{bcd}±0.29	5.22^{e}±0.22
		2.5	3.73^{j}±0.35	1.43^{h}±0.25	4.32^{i}±0.13
	Lsd(0.05)		0.25	0.18	0.20
	CV		5.6%	9.4%	4.3%

Means having the same superscript letters in the same column are not significantly different at 5% probability level

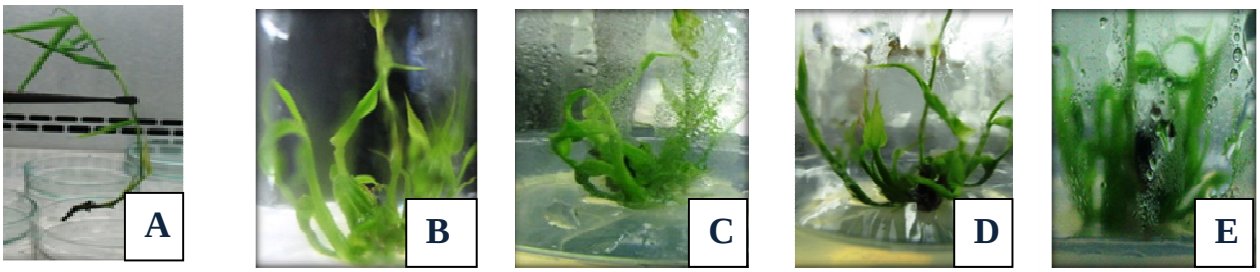

Figure 2. Response of shoots to the MS medium containing different combinations of BAP and Kin concentrations at different stages of multiplication of *O. abyssinica*: A) PGR free at the 1st stage. B) MS + 2.5 mgl^{-1} BAP +1.5mgl^{-1} Kin in the 1st stage. C) MS + 2.5 mgl^{-1} BAP +1.5mgl^{-1} Kin in the 4th stage. D) MS +1.5 mgl^{-1} BAP +1.5mgl^{-1} Kin in the 1st stage. E) MS + 1.5mgl^{-1} BAP +1.5mgl^{-1} Kin in the 3rd stage.

At the same time, some shoots obtained from the initiation media were evaluated for multiplication, elongation and preliminary rooting at various concentrations and combinations of BAP, GA_3 and NAA. ANOVA has shown that, the levels of hormones and their interactions were statistically significant for mean number of shoots per plant and shoot length. However, the interaction of BAP and NAA was insignificant for both parameters (Appendix Table 5). No preliminary roots were also observed in any hormone type and concentration.

On the other hand, the response of these hormones to multiplication and elongation was clearly lower than the results obtained from various combinations of BAP and Kinetin. The highest of which was production of 2.79 shoots per explant in the MS medium supplemented with $2.5mgl^{-1}$BAP, $2.5mgl^{-1}$ GA_3 and 0.25 mgl^{-1} NAA (Table 5). This result was only half of the number of shoots obtained from the best combination of BAP and Kinetin.

The incorporation of NAA in multiplication was believed to have a positive effect of auxin at low concentration in the culture media in that it nullifies the effect of higher cytokinin (Hu and Wang, 1983) and promotes the next step of rooting. The best performance of multiplication in the combination of BAP and NAA gave 2.29 shoots/ explant, which was lower than the results reported by Mohammed and Diab (2008).

The results of this study was consistent with other studies on micropropagation of bamboos, either BAP alone or a combination of BAP and Kin was found to give best results of shoot multiplication *in vitro* (Nadgir *et al.*,1984; Ramanayke and Yakandwala , 1997; Das and Pal, 2005; Sanjay *et al*., 2005; Kapoor and Rao, 2006). Besides the incorporation of NAA in the multiplication stage, MS medium containing low gibberellin, with lower and higher auxins was believed to promote phloem and xylem differentiation respectively. However, there were no satisfactory responses in any cases.

Table 5. Mean number of shoots/explant and shoot length in MS medium supplemented with different concentrations of BAP, GA_3 and NAA in multiplication, means are indicated as ±SD

BAP (mg/l)	GA_3 (mg/l)	NAA (mg/l)	No. of Shoots / Explant	Shoot length(Cm)
0	0	0	1.00^{j}±0.00	6.97^{a}±0.26
2.5	0	0.25	2.29^{cd}±0.25	4.92^{f}±0.26
		0.5	2.00^{fg}±0.00	5.47^{b}±0.14
		5	1.96^{fgh}±0.10	2.75^{e}±0.29
	1.5	0.25	2.00^{fg}±0.16	5.60^{b}±0.37
		0.5	1.75^{hi}±0.27	5.52^{b}±0.18
		5	1.79^{hig}±0.24	2.67^{ef}±0.40
	2.5	0.25	2.33^{bc}±0.20	5.47^{b}±0.24
		0.5	2.79^{a}±0.25	4.67^{c}±0.41
		5	2.13^{cdef}±0.21	2.53^{ef}±0.22
5	0	0.25	2.08^{def}±0.13	3.41^{d}±0.34
		0.5	2.29^{cd}±0.19	3.60^{d}±0.20
		5	1.63^{i}±0.21	2.42^{f} ±0.21
	1.5	0.25	2.54^{b}±0.10	5.51^{b}±0.28
		0.5	2.04^{ef}±0.19	5.43^{b}±0.12
		5	1.92^{fgh}±0.13	1.75^{g}±0.10
	2.5	0.25	1.62^{i}±0.14	5.65^{b}±0.10
		0.5	2.25^{cde}±0.27	4.75^{c}±0.46
		5	1.17^{j}±0.20	1.40^{h}±0.22
Lsd(0.05)			0.22	0.31
CV			9.5%	6.48%

Means having the same superscript letters in the same column are not significantly different at 5% probability level

Experiment 3. The effect of IBA on *in vitro* rooting of *O.abyssinica* (A. Rich).

In this experiment, addition of wide range of hormone concentrations (0.25-45mg/l) of the different types of rooting hormones (IBA, NAA or IAA) alone or in combinations to the MS rooting medium of different strengths (1/4 , 1/2, 3/4 and full) with or without charcoal and varying amounts of sucrose did not lead to any rooting. Instead, the shoots dried soon after inoculation in all these treatment combinations augmented with hormones. However, roots were developed in the full strength PGR free medium without charcoal. Addition of phloroglucinol (Figure 3 D) and varying media components like boric acid, calcium and iodine to the PGR free medium also showed nothing better than the rooting response obtained in the original MS medium.

ANOVA has shown a highly significant difference (P<0.00l) for the different concentrations of IBA for length of main roots, root number and percentage of roots obtained (Appendix Table 6).

Table 6. Mean number of main roots, root length and rooting efficiency in a full strength MS medium supplemented with different IBA concentrations, transformed mean are indicated as ±SD (actual mean)

IBA	No. of main roots	Root length(cm)	Shoots rooted
0	1.23^{a}±0.10(3.30)	1.12^{a} ±0.05(7.30)	1.62^{a} ±0.24 (36.7%)
0.25	1.00^{b}±0.00(0.00)	1.00^{b}±0.00(0.00)	1.00^{b} ±0.00(0.00)
25	1.00^{b}±0.00(0.00)	1.00^{b} ±0.00(0.00)	1.00^{b} ±0.00(0.00)
Lsd(0.05)	0.05	0.03	0.13
CV	5.33	3.00	11.56

Means having the same superscript letters in the same column are not significantly different at 5% probability level

The PGR free medium (Figure 3 B) showed highly significant difference in terms of mean number of main roots (3.30) from a single shoot with an average length of 7.30 cm and a rooting efficiency of 36.7% (Table 6). These results are better than the observations of Nadgir *et al.* (1984), Chaturvedi *et al.* (1993) on *B. vulgaris* and *Dendrocalamus strictus*, respectively. However, this result was less efficient than the rooting of other bamboo species like *Dendrocalamus longispathus* and *Dendrocalamus hamiltonii* as reported in Ramanayake and Yakandawala (1997)

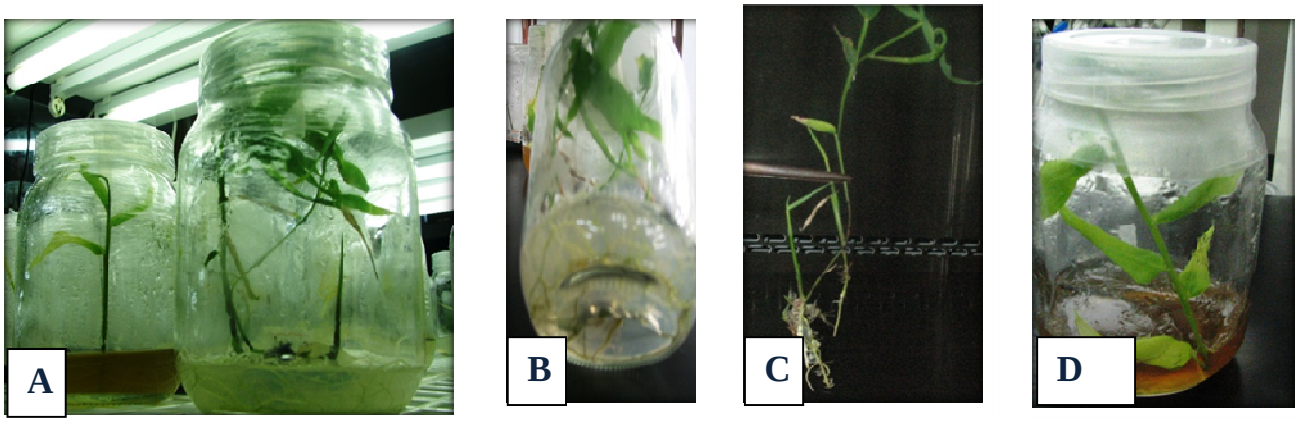

Figure 3. Rooting response of shoots in a PGR free and medium supplemented with different IBA concentrations: A) 50 mg/l IBA(former) and PGR free after 30 days of incubation. B) PGR free medium. C) *In vitro* rooted plantlet. D) PGR free medium supplemented with phloroglucinol.

Alternatively, exposing to a high concentration (50 mg/l IBA) for 3 to 10 days and then transferring to a hormone-free medium with charcoal and without charcoal also resulted in diminutive response of rooting , which was lower than the results of Ndiaye, (2006).

Therefore, the wide range of hormone types and different concentrations could not result in satisfactory level of rooting as *in vitro* rooting of bamboo could no more be hastened by the application of exogenous rooting hormones as indicated by El Nour *et al*. (1991). Availability of endogenous hormone suppressed the exogenous effect (Andrew and Bonnie, 2004).

However, *in vitro* rooting in PGR free medium followed by *ex vitro* rooting of these shoots that fail to bear roots on this medium produced interesting rise in the overall efficiency of maximum plantlet acclimatization at the same time. Hence, an average percentage of 63.3% could be attained following this procedure throughout the propagation scheme.

Experiment 4. Acclimatization of plantlets

After a month, of the 30 shoots for *in vitro* rooting and 30 shoots transferred for *ex vitro* rooting, 25 plantlets from *in vitro* rooting (Figure 4 B) and 13 from *ex vitro* rooting (Figure 3 C) were survived that account 83.3% and 43.3% acclimatization efficiency, respectively . In the acclimatization of *in vitro* rooted plantlets, this experiment resulted consistent results with the works of Sanjay and Saint, 2003 and Bist *et al.*, 2010.

Figure 4. Acclimatization of bamboo plantlets micropropagated *in vitro*; A) Survived plantlet on the tray in the 10^{th} day. B) Plantlets obtained from the *in vitro* rooting step and transferred to the plastic pots. C) Plantlets acclimatized by *ex vitro* rooting.

5. SUMMARY AND CONCLUSIONS

Bamboo, a treelike grass, is an economically, socially and ecologically useful plant in the world. It has numerous benefits ranging from construction, furniture, textile, charcoal, paper and energy up to various forms of foods and medicinal values. However, due to eventual death after flowering, the Ethiopian bamboo biodiversity is currently in danger. Hence, micropropagation could circumvent this threat and help the future commercial plantation and income generation from this sector.

Nodal explants are probably the best explant types as they can have active buds that could easily sprout during initiation in a culture medium and are easier during sterilization and preparation for culture. Sterilization of nodal explants by using 2% sodium hypochlorite solution for 10 minutes, followed by rinsing alcohol for a minute and then 1% for 10 minutes again produced up to 100% contamination free and effective initiation.

In active months (October-January), explants could be initiated in a week especially if BAP ($2.5mgl^{-1}$ in MS medium) is supplemented. Plant growth regulator free MS medium also produced initiation of these explants but subsequently the shoots become taller without any further auxiliary buds that can further support in the next stage of multiplication. Multiple nodes from a shoot in tissue culture media pose difficulty to establish themselves than the efficiency of multiple auxiliary buds that can proliferate from a single explant. Hence, incurring small amount of BAP ($2.5\ mgl^{-1}$) can produce strongest multiple shoots even in the stage of initiation

In vitro multiplication, which is the most critical step in mass propagation, can be done by establishing 2 cm long shoots in a medium supplemented with BAP and Kinetin. Within three weeks, greater than fivefold (5.9) shoots was achieved from a single explant in the 1^{st} stage of multiplication prior to sub culture in MS medium supplemented with $2.5mgl^{-1}$ BAP and $1.5\ mgl^{-1}$ Kinetin.

However, the combinations of hormones tend to accumulate in the shoots in successive stages of subculture. Hence, the MS medium supplemented with $1.5\ mgl^{-1}$ BAP and $1.5\ mgl^{-1}$ Kinetin resulted in better response (5.68) of multiple shoot production from a single explant after the 3^{rd} stage of subculture. Therefore, the first two stages of multiplication

should be supplemented with 2.5mgl^{-1} BAP and 1.5 mgl^{-1} Kinetin and the next two consecutive steps should be augmented with the combination of 1.5 mgl^{-1} BAP and 1.5 mgl^{-1} Kinetin in the MS multiplication medium.

Up on three subculture times, more than 1000 explants can be obtained from a single shoot inoculated in to multiplication medium in three months of time. Having in mind that, at least two plants with independent auxiliary bud are produced from a single explant from initiation, more than 2000 shoots could be ready for rooting from that single nodal bud of initiation. Multiplied shoots should be kept in growth free medium for at least 15 days so as to reduce the inhibiting effect of cytokinines in rooting.

In vitro rooting of bamboo shoots is one of the processes that posed difficulty in the effectiveness of bamboo mass propagation programs. In this study, plantlets rooted in a full strength MS medium. The roots were long (>7cm) and at least three main roots in a single plant were obtained in the PGR free medium. In this case, the efficiency of rooting was 36.7%. Addition of rooting hormones in to the PGR free full strength MS medium caused drying of shoots. On the other hand, modification of other MS media constituents such as phloroglucinol, boric acid, calcium and iodine, which are believed to increase root sprouting, could not improve the rooting percentage.

Direct *ex vitro* rooting of the shoots that fail to bear roots under *in vitro* conditions was also another possibility utilized as alternative in order to acclimatize maximum possible plantlets. Hence, more than 43% *ex vitro* rooting and survival rate was acquired. This result alarms that, some components of MS medium other than rooting hormone need to be modified and further studied. Hence, the whole experiment of the final rooting frequency reaches at least 63.3% from the shoots in a single consecutive experiment.

Young plantlets should be maintained in a greenhouse condition, where the plantlets are kept in the trays covered with plastics to maintain humidity. Hence, 83.3% of the *in vitro* rooted and 43.3% of the *ex vitro* rooted plantlets (which were acclimatized simultaneously with the rooting step) were ready for field planting.

In conclusion, bamboo tissue culture could be one potential option in order to control the ever devastating loss of bamboo biodiversity currently happening in Ethiopia. It could save

the lives dependent on bamboos and start getting sustainable returns from this solid business. Besides this, this plant is a source of main instruments and cultural figures of Ethiopian restaurants and recreation centers with a modern style. Thus, micro propagation of *O.abyssinica* not only helps the biodiversity from loss or furnishes the green environment; it can substantially help in fabricating genetically uniform, vigorous and sustainable supply of plantlets for commercialization that proponent the national income and international market.

The biology of bamboo plant is something astonishing that requires close scrutiny. Bamboo is a woody grass that has a nature of grasses and properties of woody plants. Due to this case, it dies soon after flowering like any other members of the grass family. On the other hand, it needs too many decades to flower like any other woody plants. As a result, *in vitro* direct regeneration is a first step to clutch the plant in laboratory conditions. The successive experiments of protocol optimization in this study have resulted in promising results for mass propagation bamboos under *in vitro* conditions.

Therefore, the protocol for mass propagation should be started by inoculation of nodal explants in MS medium supplemented with 2.5mgl^{-1} BAP for initiation followed by multiplication of shoots by adding 2.5mgl^{-1} BAP and 1.5mgl^{-1} Kinetin for the first two stages of culture growth and 1.5 mgl^{-1} BAP and 1.5 mgl^{-1} Kinetin for the 3rd and 4th stages of culture growth. *In vitro* rooting of shoots in full strength PGR free MS medium and *ex vitro* rooting of the shoots that fail to beer roots *in vitro* is preferable way of getting maximum percentage of acclimatized plantlets. Acclimatization should be done in glass house by covering the plantlets with plastic soon after their discharge from the laboratory.

For the future, the seasonal effects of bud sprouting in bamboo mass propagation scheme should be further studied in Ethiopian conditions. On the other hand, the *in vitro* rooting needs further refinement on basal medium to improve the rooting percentage. Somatic embryogenesis from different explants should be tested. Once *in vitro* regeneration methods are optimized, further works regarding bamboo biology and biotechnology, specially gene transfer via agrobacterium rhizogenes, sequencing and transfer of rooting gene and metabolic profiling for genetic improvement studies of flowering should go forward.

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7. APPENDIX

Appendix 1. The media composition and ANOVA tables

Appendix Table 1. Composition of MS medium

MURASHIGE and SKOOG (MS) (1962) media

Code	Nutrient	Stock solution (g)		Volume of stock for 1L Full MS media
MS1	Ammonium nitrate (NH_4NO_3)	33.0	In 1000 ml	50 ml
	Potassium nitrate (KNO_3)	38.0		
MS2	Magnesium sulphate ($MgSO_4.7H_2O$)	18.07	In 500 ml	5 ml
	Manganese sulphate ($MnSO_4.H_2O$)	1.69		
	Zinc sulphate ($ZnSO_4.7H_2O$)	0.86		
	Copper sulphate ($CuSO_4.5H_2O$)	0.0025		
MS3	Calcium chloride ($CaCl_2.2H_2O$)	33.22	In 500 ml	5 ml
	Potassium iodide (KI)	0.083		
	Cobalt chloride ($CoCl.6H_2O$)	0.0025		
MS4	Potassium dibasic phosphate (KH_2PO_4)	17.0	In 500 ml	5 ml
	Boric acid (H_3BO_3)	0.62		
	Sodium molbdate ($Na_2MoO_4.2H_2O$)	0.025		
MS5	Na_2EDTA	3.726	In 500 ml	5 ml
	Iron sulphate ($FeSO_4.7H_2O$)	2.78		
MS6	Myo-inositol	10.0	In 500 ml	5 ml
	Glycine	0.2		
	Thiamine HCl	0.01		
	Pyridoxine HCL	0.05		
	Nicotinic acid	0.05		

- In preparation of stock solutions **each nutrient should completely dissolve separately** in distilled water before adding the next nutrient then the final volume should be adjusted by adding distil water.
- During preparation of **MS5;** Na_2 EDTA and iron sulphate should be dissolved in hot water separately and after dissolving add iron solution over EDTA: **NEVER THE OPPOSITE WAY**.

Plant growth regulators (PGRs) chemical specification

- When preparing PGRs: they should be prepared in a ratio of 1mg : 1ml
- To get PGRs dissolved: add few drops (4-5) of specified solvent after dissolve completely adjust the final volume by adding distill water

PGRs	Solvent
BAP, BA, 2ip, IBA, TDZ, Kinetin, NAA, Zeatin	NaOH (1N)
GA3,	EtOH

Appendix Table 2. ANOVA summary for the effect of BAP on *in vitro* initiation of *O.abyssinica*

Dependent Var.	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Days to initiation	BAP	6	20.22059524	3.37009921	102.69	<.0001
	Error	35	1.14863333	0.03281810		
	Total	41	21.36922857			
% Regeneration	BAP	6	3062.264260	510.377377	2.55	0.0371
	Error	35	6992.79247	199.79407		
	Total	41	10055.05673			
Shoots per explant	BAP	6	6.60142857	1.10023810	86.62	<.0001
	Error	35	0.44458333	0.01270238		
	Total	41	7.04601190			
Nodes per shoot	BAP	6	11.52202381	1.92033730	117.83	<.0001
	Error	35	0.57041667	0.01629762		
	Total	41	12.09244048			
Shoot length	BAP	6	31.32224762	5.22037460	171.43	<.0001
	Error	35	1.06580000	0.03045143		
	Total	41	32.38804762			

	R-Square	Coeff Var	Root MSE	Mean
Days to initiation	0.946248	3.785384	0.181158	4.785714
Percentage of regeneration	0.304550	15.29202	14.13485	92.43286
Shoots per explants at initiation	0.936903	8.084720	0.112705	1.394048
Nodes per shoot at initiation	0.952829	6.774238	0.127662	1.884524
Length of shoots at initiation	0.967093	4.182823	0.174503	4.171905

Appendix Table 3. ANOVA summary of effect of BAP and Kinetin on *in vitro* multiplication of *O.abyssinica* at the first stage of culture growth

	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Shoots per explant	BAP	4	172.0107000	43.0026750	1021.85	<.0001
	KIN	3	133.5205500	44.5068500	1057.59	<.0001
	BAP*KIN	12	54.6437000	4.5536417	108.21	<.0001
	Error	180	7.5750000	0.0420833		
	Total	199	367.7499500			
Nodes per shoot	BAP	4	11.92030000	2.98007500	104.58	<.0001
	KIN	3	7.15615000	2.38538333	83.71	<.0001
	BAP*KIN	12	20.15210000	1.67934167	58.94	<.0001
	Error	180	5.12900000	0.02849444		
	Total	199	44.35755000			
Shoot length	BAP	4	77.08630000	19.27157500	222.22	<.0001
	KIN	3	17.49993750	5.83331250	67.26	<.0001
	BAP*KIN	12	32.94350000	2.74529167	31.66	<.0001
	Error	180	15.6102500	0.0867236		
	Total	199	143.1399875			

	R-Square	Coeff Var	Root MSE	Mean
Shoots per explant	0.979402	7.391181	0.205142	2.775500
Nodes per shoot	0.884371	7.490702	0.168803	2.253500
Shoot length	0.890944	5.788761	0.294489	5.087250

Appendix Table 4. ANOVA summary of effect of BAP and Kinetin on *in vitro* multiplication of *O.abyssinica* at different stages

Dependent Var.	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Shoots per Explant	STAGE	3	1.70018750	0.56672917	7.33	0.0001
	BAP	1	0.33306250	0.33306250	4.31	0.0397
	KIN	1	39.10506250	39.10506250	505.71	<.0001
	STAGE*BAP	3	6.92468750	2.30822917	29.85	<.0001
	STAGE*KIN	3	9.02568750	3.00856250	38.91	<.0001
	BAP*KIN	1	9.16806250	9.16806250	118.56	<.0001
	STAGE*BAP*KIN	3	3.60068750	1.20022917	15.52	<.0001
	Error	144	11.13500000	0.07732639		
	Total	159	80.99243750			
Nodes per Shoot	STAGE	3	0.92925000	0.30975000	7.61	<.0001
	BAP	1	1.02400000	1.02400000	25.16	<.0001
	KIN	1	10.20100000	10.20100000	250.67	<.0001
	STAGE*BAP	3	1.18850000	0.39616667	9.74	<.0001
	STAGE*KIN	3	1.17650000	0.39216667	9.64	<.0001
	BAP*KIN	1	0.07225000	0.07225000	1.78	0.1848
	STAGE*BAP*KIN	3	0.56225000	0.18741667	4.61	0.0041
	Error	144	5.86000000	0.04069444		
	Total	159	21.01375000			
Shoot length	STAGE	3	7.32150000	2.44050000	48.72	<.0001
	BAP	1	7.56900000	7.56900000	151.09	<.0001
	KIN	1	36.29025000	36.29025000	724.40	<.0001
	STAGE*BAP	3	1.01650000	0.33883333	6.76	0.0003
	STAGE*KIN	3	0.79625000	0.26541667	5.30	0.0017
	BAP*KIN	1	0.93025000	0.93025000	18.57	<.0001
	STAGE*BAP*KIN	3	1.64625000	0.54875000	10.95	<.0001
	Error	144	7.21400000	0.05009722		
	Total	159	62.78400000			

	R-Square	Coeff Var	Root MSE	Mean
Shoots per explant	0.862518	5.625515	0.278076	4.943125
Nodes per shoot	0.721135	9.410082	0.201729	2.143750
Shoot length	0.885098	4.255211	0.223824	5.260000

Appendix Table 5. ANOVA summary of effect of BAP, GA_3 and NAA on in vitro multiplication, elongation and preliminary rooting of *O.abyssinica*

Dependant Var.	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Number of shoots/Explant	BAP	2	6.80860136	3.40430068	96.42	<.0001
	GA	2	0.51412385	0.25706193	7.28	0.0011
	NAA	3	9.98336988	3.32778996	94.25	<.0001
	BAP*GA	2	4.62939467	2.31469733	65.56	<.0001
	GA*NAA	4	2.67684837	0.66921209	18.95	<.0001
	BAP*NAA	1	0.00000000	0.00000000	0.00	1.0000
	BAP*GA*NAA	4	0.90879978	0.22719994	6.43	0.0001
	Error	95	3.35416667	0.03530702		
	Total	113	23.57017544			
Shoot length	BAP	2	57.4021832	28.7010916	382.95	<.0001
	GA	2	2.1370906	1.0685453	14.26	<.0001
	NAA	3	226.4690351	75.4896784	1007.24	<.0001
	BAP*GA	2	10.6536501	5.3268250	71.07	<.0001
	GA*NAA	4	24.6029094	6.1507273	82.07	<.0001
	BAP*NAA	1	0.0000000	0.0000000	0.00	1.0000
	BAP*GA*NAA	4	0.8707943	0.2176986	2.90	0.0257
	Error	95	7.1200000	0.0749474		
	Total	113	282.3407018			

	R-Square	Coeff Var	Root MSE	Mean
No. of shoots/explant	0.857694	9.499239	0.187902	1.978070
Shoot length (cm)	0.974782	6.483014	0.273765	4.222807

Appendix Table 6. ANOVA summary of effect of IBA on *in vitro* rooting of *O.abyssinica*

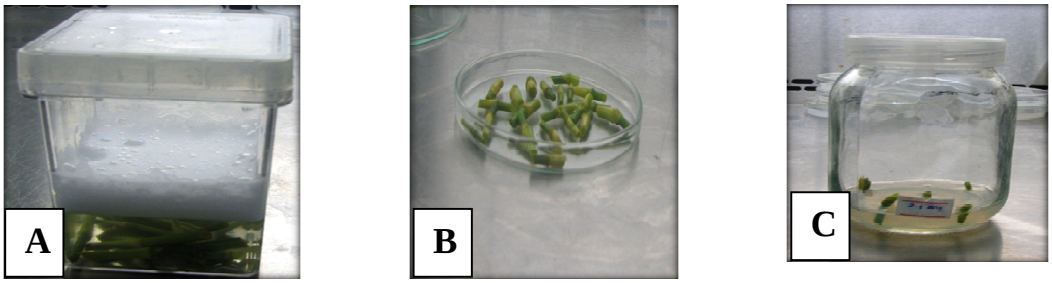
Dependent Var.	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Number of Main roots	IBA	2	0.34854187	0.17427093	53.02	<.0001
	Error	27	0.08874279	0.00328677		
	Total	29	0.43728465			
Root length	IBA	2	0.09750022	0.04875011	50.00	<.0001
	Error	27	0.02632548	0.00097502		
	Total	29	0.12382570			
Percentage of Rooting	IBA	2	2.58500206	1.29250103	66.30	<.0001
	Error	27	0.52634461	0.01949424		
	Total	29	3.11134666			

	R-Square	Coeff Var	Root MSE	Mean
Number of main roots	0.797059	5.327026	0.057330	1.076217
Root length (cm)	0.830831	11.56226	0.139622	1.207565
Percentage of rooting	0.787399	3.001532	0.031225	1.040311

Appendix 2. Mother plant preparation explants preparation and inoculation of nodal cultures.

Appendix Figure 1. One year old mother plants of *O.abyssinica* grown in a glasshouse.

Appendix Figure 2. Preparation and inoculation of explants.



A) Surface sterilization of explants with 2% NaClO and two drops of tween 20 solution. B) Explants ready for inoculation. C) A culture vessel containing 5 explants soon after culture.