

**MICROPROPAGATION OF SOME EDIBLE BAMBOO SPECIES AND
MOLECULAR CHARACTERIZATION OF THE REGENERATED
PLANTS**

*A thesis submitted in fulfillment
of the requirement for the award of the degree of*

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in the thesis entitled “**Micropropagation of some edible bamboo species and molecular characterization of the regenerated plants**” in fulfillment of the requirement for the award of the **Degree of Doctor of Philosophy** at the Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period from July 2007 to April 2014, under the supervision of Dr. Manju Anand, Assistant Professor, Department of Biotechnology, Thapar University, Patiala and Dr. Anil Sood, Chief Scientist and Head, Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur (HP). The report has not been submitted for the award of any other degree or certificate in this or any other University.



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Certified that the thesis entitled "Micropropagation of some edible bamboo species and molecular characterization of the regenerated plants" submitted by Ms. Jasmine Brar, in fulfillment of the requirement for the award of **Degree of Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by her under our supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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Dedicated to my Parents
for their endless Love, Support
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Encouragement

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ABSTRACT

The present study was conducted on three important edible bamboo species namely *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos* which have been listed as priority species by International Union for Conservation of Nature (IUCN) with an aim to establish an efficient and reproducible protocol for their large scale multiplication under *in vitro* conditions. The other objective was to assess the genetic fidelity of *in vitro* raised plants by molecular markers and to study their field performance. A comparative evaluation of various physiological and biochemical parameters was carried out to compare *in vitro* plants with hardened *ex vitro* plants.

Different explants like nodal explants, apical buds and leaves were taken from precocious branches of field grown, healthy plants for initiating aseptic cultures in all the three species. Among the various explants tested, unexpanded buds from secondary and tertiary branches were found to be the best for raising cultures as they responded favourably to different media combinations besides being easier to handle. The collection time of explant for culture initiation greatly influenced the frequency of bud break and number of shoots produced. Explants collected during spring in the months of February to April gave best response in terms of increased bud break (> 90%), early shoot initiation and decreased contamination. Rainy season (June to August) depicted more than 80% bud sprouting but was not a preferred season as it had a direct influence on contamination rate (95%) and survival percentage of explants. Likewise, larger explants (more than 25mm) had a direct effect on culture initiation and took least time for bud sprouting.

Bacterial contamination was the major problem encountered during the initiation of aseptic cultures which was solved by treating explants with streptomycin sulphate and tetracycline (0.02% each) in addition to sodium hypochlorite (15%) and mercuric chloride (0.1%) while carrying out disinfection. An efficient procedure for multiple shoot proliferation from axillary buds was achieved on cytokinins supplemented media. In *D. membranaceus* and *B. bambos*, best response for multiple shoots induction was achieved on Murashige and Skoog's (1962) medium containing 4.4 μM of 6-Benzylaminopurine (BAP) alongwith 1.16 μM of Kinetin (Kn) forming 13.40 ± 1.5 and 21.70 ± 2.40 shoots respectively. In *B. balcooa*, highest shoot proliferation (19.8 ± 1.4 shoots) was obtained when 4.4 μM of BAP was used in conjunction with 0.53 μM of Naphthalene Acetic Acid (NAA). Once the clusters of shoots were formed, small clumps of 3-4 shoots were excised and transferred onto fresh multiplication medium every 4 weeks for continuous shoot proliferation.

Rooting is a major bottleneck while carrying out *in vitro* multiplication of bamboos. For root induction, *in vitro* raised shoots were divided into clumps of 3-4 shoots and transferred onto MS medium containing different auxins. In *D. membranaceus*, half strength MS medium containing 5.37 μM of NAA in conjunction with 4.4 μM of BAP gave the best rooting percentage of 65%. In *B. balcooa*, 76.6% rooting was observed on full MS medium supplemented with 16.11 μM of NAA. In the present investigation on *B. bambos*, rooting was difficult to achieve and has been a major problem to be worked out. Best rooting response of 60% was achieved on half strength MS medium supplemented with 9.80 μM Indole-3 Butyric Acid (IBA). An initial pulse treatment of coumarin (9 mg/l) for 10 days and then shifting the cultures to coumarin free medium improved the rooting percentage by 15 to 20%. In the present study, IBA and NAA proved effective for providing initial stimulus for *in vitro* rooting but advanced root growth was noticed only on transfer to auxin free medium. Propagule size used for recurrent multiplication of shoots as well as *in vitro* rooting was a crucial factor during present study. Shoot clumps each having three shoots rather than a single shoot were found to be most effective for shoot proliferation and *in vitro* rooting.

In vitro raised plants of *D. membranaceus*, *B. balcooa* and *B. bambos* were carefully rescued from the vessels and were initially transferred to the plastic pots containing moist riverbed sand and covered with perforated plastic jars/covers to maintain high internal humidity and were kept for a period of 14 days in the growth room. Thereafter, they were transferred onto potting mixture containing sand: soil: farmyard manure (1:1:1) and shifted to green house under controlled conditions of low radiance and high RH where they depicted 67% survivability in *D. membranaceus*, 92.5% in *B. balcooa* and 80% in *B. bambos*. Tissue culture raised plants of all the three species were initially acclimatized in green house and retained there for 5-6 months. In this period, the plants developed an efficient root system, built up new leaves and became photosynthetically active. The best time for their transfer to the fields was the beginning of rainy season i.e. June end or July when they exhibited 100% survival. Hardened plants were successfully transplanted in the field to pits (2ft \times 2ft \times 2ft) at a plant to plant and row to row distance of 6 meters and successfully established under field conditions. Plants showed well developed root and shoot systems with no phenotypic variations observed when compared to the mother plant. Their field performance after six months was recorded by studying different plant parameters like height, number of shoots, number of leaves, number of axillary shoots per clump and number of internodes per shoot formed. *D. membranaceus* registered a maximum growth in terms of height achieved i.e. 101.66 cm, 294 leaves formed and on an average 24.6 new shoots emerged during this

period. *B. balcooa* showed maximum height of 95.5 cm, 255 leaves and 20 new shoots formed while in *B. bambos*, height of 50 cm was achieved with 94 leaves and 12 new shoots formed. This increase in the overall growth parameters has demonstrated that plants acclimatized well in the field conditions where they are thriving very well.

For callus induction, *in vitro* raised shoots were excised into small pieces (0.5-1cm) and transferred onto MS medium supplemented with different auxins and cytokinins. In *D. membranaceus* and *B. bambos*, callus was initiated on MS medium supplemented with 5.37 μM of NAA and 4.53 μM of 2, 4-Dichlorophenoxyacetic Acid (2,4-D) alongwith 3% sucrose when incubated in the dark. Calli formed were of two types-fast growing mucilaginous and comparatively slow growing, nodular and compact calli. In *D. membranaceus*, differentiation of shoot buds from callus was observed on MS medium supplemented with 4.53 μM of 2, 4-D and 5.37 μM of NAA. However further elongation of shoots and formation of leaves occurred when transferred onto medium supplemented with BAP (4.4 μM) in conjunction with Kn (1.15 μM). In *B. bambos*, callus was found to be rhizogenic with 40% callus lumps showing the differentiation of roots on lower concentration of 2,4-D (2.26 μM) in conjunction with NAA (1.14 μM). The callus showed the development of green protuberances on BAP (2.2 μM) alongwith 2,4-D (4.53 μM) but these did not grow into shoots on any of media combinations tried. Histological studies revealed the formation of globular meristemoids from the nodular callus which developed into shoot bud primordia.

An *in vitro* propagation protocol using aged seeds of *D. membranaceus* was successfully established. The effect of sterilants, light conditions, exogenous application of plant growth regulators and temperature in overcoming germination barriers in ageing seeds were studied. It was found that mercuric chloride (0.1%) along with bleach (15%) was more effective in raising aseptic cultures. Dark conditions, high temperature around 30°C and soaking of seeds in Gibberellic Acid (GA_3) solution overnight (50 ppm) stimulated high percentage of seed germination with corresponding increase in shoot length (2.7 ± 0.7 mm) and number of sprouts (2.1 ± 0.7) per explant during culture initiation. BAP (8.8 μM) acted synergistically with Kn (2.3 μM) to give optimum germination percentage of $70.0 \pm 13.9\%$ as compared to 63% when used individually. Biochemical test confirmed the presence of a starchy endosperm in seeds which was consumed for emergence of radicle during germination process. Loss of viability and vigour of seeds of *D. membranaceus* after a year was confirmed by tetrazolium chloride test.

Out of the different auxins tried for callus induction from seeds in *D. membranaceus*, best callusing occurred on 4.53 μM of 2, 4-D and 5.37 μM of NAA supplemented medium forming a mass of nodular callus after 40 to 45 days capable of sustained growth on repeated subculturing. When callus was shifted to 2,4-D (4.87 μM) in conjunction with BAP (1.13 μM), nearly 40% callus lumps showed development of green bud like protuberances but these did not grow into shoots despite continuous culturing on the same medium.

There was a complex web of signal interactions between sucrose concentration and the growth of the callus tissue in *D. membranaceus*. Best results were obtained on 1% sucrose with a relative growth rate (RGR) of 0.46 ± 0.05 mg/g/day. Strength of basal MS medium was found to have an effect on RGR of the callus with highest RGR (0.45 ± 0.02 mg/g/day) of callus observed on $\frac{1}{2}$ strength MS medium. The application of GA₃ (2.8 μM) was found to have an effect on the growth rate depicting a value of i.e. 0.63 ± 0.05 mg/g/day. Myo-Inositol at a concentration of 300 mg/l gave the best RGR giving the highest value of 0.49 ± 0.05 mg/g/day.

A comparative evaluation of 6 weeks old *in vitro* plants and 6 months old hardened field grown plants was made with respect to various physiological and biochemical parameters. In all the three species, electrolyte leakage percentage was found to be higher in *ex vitro* raised plants whereas relative water content percentage was higher in the *in vitro* grown plants. Analysis of total soluble sugars and starch content indicated *in vitro* plants to be richer in carbohydrate reserves. However, proline and lignin content, stomatal density and leaf area index were greater under *ex vitro* conditions. Maximum values of chlorophyll content and carotenoids were observed under *ex vitro* conditions when acetone was used as a solvent. In all the three species, the anatomical studies of culm, leaf and root of *in vitro* plants did not show any major difference in their basic structure when compared to the field grown plants except for the presence of more lignified tissue in the latter.

For ascertaining the clonal fidelity of tissue culture raised plants following 6 months of their hardening, 15 randomly selected plants and the mother plant were subjected to RAPD and ISSR analyses. In *D. membranaceus*, out of 35 scanned Random Amplified Polymorphic DNA (RAPD) markers, only 30 primers were informative as they produced 73 amplified products. OPA series gave the best amplification followed by OPO and OPT series. During Inter Simple Sequence Repeats (ISSR) analysis of 20 markers, only 15 ISSR primers gave 38 scorable bands. UBC 834 gave 5 bands followed by UBC 840 giving 4 bands and UBC 857, UBC 850 and UBC 888 giving 3 bands each. In *B. balcooa*, out of 25 RAPD markers scanned, only 21 primers produced 61 amplicons. OPO series gave the best amplification

followed by OPA and OPT series. In case of ISSR analysis using 15 markers only, 10 ISSR primers gave 28 scorable bands. UBC 810, 811 and 888 gave maximum amplified products in the range of 250 to 1500 bp. Similarly, in *B. bambos* out of 15 RAPD markers scanned, only 10 primers produced amplicons with OPA series giving the best amplification. In case of ISSR analyses using 10 markers, only 5 markers gave scorable bands with UBC 818 giving the best amplification products with sharp bands in the range of 200 to 1400 bp. All banding profiles from the micropropagated plants were monomorphic and similar to those of mother plant, thereby, confirming true to type nature of *in vitro* raised plants.

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Objective 1: To develop and standardize an efficient, reliable and reproducible protocol for mass propagation of *Bambusa bambos*, *Bambusa balcooa* and *Dendrocalamus membranaceus* under *in vitro* conditions.

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Objective 2: Checking their clonal fidelity by RAPD techniques to ensure quality and to study the field performance of the *in vitro* raised plants.

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ABBREVIATIONS

2,4 -D	2,4 - Dichlorophenoxy Acetic Acid
2,4,5-T	2,4,5-Trichlorophenoxy Acetic Acid
2-ip	2-Isopentyl Adenine
μ	Micron
%	Percent
ANOVA	Analysis of Variance
Ads	Adenine Sulphate
AFLP	Amplified Fragment Length Polymorphism
BAP	6-Benzylaminopurine
bp	Base Pair
°C	Degree Celsius
CRBD	Complete Randomized Block Design
Chl	Chlorophyll
Cm	Centimeter
CO ₂	Carbon Dioxide
CM	Coconut Milk
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DPX	Disterene Dibutylphthalate Xylene
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
dNTPs	deoxy Ribonucleoside Triphosphates
EDTA	Ethylene Diaminetetraacetic Acid
EL	Electrolyte Leakage
fr.wt.	Fresh Weight
FAA	Formalin Acetic Acid Alcohol
FAO	Food and Agriculture Organization
FYM	Farmyard Manure
GA ₃	Gibberellic Acid
GAA	Glacial Acetic Acid
H ₂ SO ₄	Sulphuric Acid
Hr	Hour

IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
IHBT	Institute of Himalayan Bioresource Technology
INBAR	International Network for Bamboo and Rattan
IPGRI	International Plant Genetic Resource Institute
ISTA	International Seed Testing Association
IUCN	International Union for Conservation of Nature
Kb	Kilo Basepair
LAI	Leaf Area Index
M	Molar
masl	Meters Above Sea Level
MgCl ₂	Magnesium Chloride
min	Minutes
ml	Milliliter (s)
mM	Millimolar
MS	Murashige and Skoog's (1962) Medium
μl	Microlitre
μM	Micromole
N	Normal
NAA	Naphthalene Acetic Acid
NaOCl	Sodium Hypochlorite
ng	Nano-gram
Nm	Nano-meter
NPP	Net Primary Productivity
OD	Optical Density
OP	Operon Technologies
PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulator
ppm	Parts Per Million
PPFD	Photosynthetic Photon Flux Density
PVP	Polyvinyl Pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RGR	Relative Growth Rate
RNA	Ribonucleic Acid

rpm	Rotation Per Minute
RWC	Relative Water Content
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SSR	Simple Sequence Repeats
T _a	Annealing Temperature
<i>Taq</i>	<i>Thermus aquaticus</i>
TBA	Tertiary Butyl-Alcohol
TC	Tissue Culture
TDZ	Thidiazuron
TE buffer	Tris-EDTA Buffer
T _m	Melting Temperature
TSS	Total Soluble Sugars
TTC	Triphenyl Tetrazolium Chloride
UBC	University of British Columbia
UV	Ultra-Violet
UNEP	United Nations Environment Programme
Vis	Visible
w/v	Weight/Volume
v/v	Volume/Volume
WSD	Water Saturation Deficit

1. Introduction

1.1 Bamboos: An Overview

Bamboos represent one of the world's most important natural and renewable bioresources and are among the most useful plants known to mankind. Bamboos are a group of evergreen perennial grasses belonging to family - Poaceae and sub-family - Bambusoideae. They constitute a major part of the natural vegetation of tropical, sub-tropical and wild temperate regions. There are about 88 genera and 1400 species of bamboos distributed worldwide covering an area of more than 14 million hectares with 80% of species and area under bamboos confined to south and south-east Asia, largely in China, India and Myanmar (Wu and Raven, 2006). China has the richest bamboo resource having 34 genera and 539 species and largest bamboo industry worldwide having a total of about 5 million hectare of bamboo forests (Maoyi, 1998 and Bystriakova et al. 2004). India is second after China in bamboo resources having 125 species belonging to 23 genera and produces 4-6 million tonnes of bamboos annually, out of which 2.2 million tonnes is used in the paper industry (Sharma 1980 and Gillis et al. 2007). Other nations with significant bamboo production are Bangladesh, Indonesia and Thailand.

Bamboos have been an integral part of human culture and the world's economy for millennia. They are capable of providing ecological, economic and livelihood security to countless people. Bamboos form the backbone of the rural economy of many south-east Asian countries including India sustaining the livelihood of millions of people and are often referred to as "poor man's timber" (Yeasmin et al. 2014). Bamboos are highly versatile and multipurpose plants having more than 1500 commercial applications and are rightly called "green gold" of forests. According to an estimate, 2-5 billion people depend on or use bamboo materials to a value of 7 million US \$ per annum (Liese, 1992).

Bamboos are having largest use in paper and pulp industry (Varmah and Pant, 1981) and in India, it constitutes nearly 70% of the raw material for this industry (Rout and Das, 1994). Besides their application in paper industry, they are extensively used as timber for house building, furniture making, daily sundry goods, agriculture and fisheries tools and crafting materials. Leaves serve as an excellent cattle fodder especially during winters when there is shortage of animal fodder (Sahoo et al. 2010). Bamboo shoots are one of the most widely consumed delicacies in many countries including China, Japan, Thailand, Malaysia, Singapore, Hongkong, Indonesia and northeastern parts of India (Tripathi, 1998). Shoots can be consumed fresh, cooked, pickled, fermented and canned. The shoots are rich in proteins

and fiber contents and are known to lower cholesterol (Nirmala et al. 2008). Additionally, new products such as bamboo charcoal, bamboo vinegar, bamboo juice and bamboo fiber products have been developed. Besides, their immense economic applications, bamboos render many ecological services as well. Growing as much as a meter per day, bamboo is used to stabilize and remediate eroded and environmentally degraded landscape (Ogunwusi and Jolaoso, 2012). The importance of bamboo forests as a potential modulator of global environment has been proposed (Bystriakova et al. 2004). As atmospheric oxygen pump, bamboos produce 35% more oxygen than deciduous trees (Titeux, 2011). Bamboos serve as efficient agents for erosion control and conservation of water and soil (Maoyi, 1998). In the recent years, bamboos have gained importance in social forestry programmes due to their short rotation cycles, fast growth and possibility of their being harvested on sustainable basis (Godbole, 2003).

1.2 Growth and Development of Bamboos

Bamboos are evergreen monocotyledonous plants belonging to 'Angiospermae', the group of flowering plants. Bamboos comprise a sub family Bambusoideae of grasses (Poaceae) belonging to tribe Bambuseae. It is further divided into many genera and species based upon growth and morphology as depicted in [Figure 1.2](#). The tree forms of bamboos include *Bambusa balcooa*, *Dendrocalamus hamiltonii* etc; reed forms include *Ochlandra* species, *Melocanna* species belongs to straggler forms and shrub forms include *Arundinaria* species. Bamboos are mostly polyploids and natural populations are highly heterogenous and heterozygous (Mehra and Sharma, 1975). Most tropical bamboos are hexaploids ($2n=6x=72$) while most temperate bamboos are tetraploids ($2n=4x=48$), assuming a basic chromosome number of $x=12$ (Kellogg and Watson, 1993 and Clarke et al. 1995).

Bamboo stem emerges from the ground at its full girth and grows to its full height in a single growing season. Vascular cambium is absent in bamboos and hence no annual rings are seen in the bamboo culms. The absence of secondary growth wood causes the stem of bamboos to be columnar rather than tapering (Xiaohongl and Yulong, 2005). Morphologically, shoot has a distal aerial part called the culm, a proximal ground level part called culm neck and a subterranean part called the rhizome. Culm consists of nodes and internodes. Nodes consist of meristematic tissue from where culm sheath and a branch arise.

Rhizome: The rhizome is the underground part of the bamboo plant consisting of nodes and internodes bearing roots arising from the nodes. There are two broad types of rhizome:

1. Pachymorph (Sympodial) rhizome: A clump forming rhizome in which each axis is dominant and secondary axes (culms) developing from it e.g. species belonging to genera such as *Dendrocalamus* and *Bambusa*. 2. Leptomorph (monopodial) rhizome: A non clump forming rhizome which grows horizontally and secondary axes (culms) develop from it as lateral buds e.g. species belonging to genus *Phyllostachys*. These differences in rhizome system can be regarded as adaptation to climatic conditions to which bamboos are native to: with monopodial bamboos being native to temperate climate with cool, wet winters and sympodial bamboos to tropical climate with a pronounced dry season (Stapleton, 1994 and FAO, 2005). The tight-clumping habit of tropical species supposedly evolved from the leptomorph form of rhizome which provides less rhizome surface to dehydrate during extended dry seasons (Farrelly, 1984).

Shoot: It is an emerging young bamboo culm. It originates from the buds of the rhizome. Shoots are harvested at the time of emergence or when culms are young. It can be eaten and is a good source of nutrition and fiber (Chongtham et al. 2011).

Culms: Culm originates from rhizome as a bud, emerges from the ground as a shoot, and then elongates to become a culm. Initially tender, the shoot ultimately grows into a woody culm. An individual culm attains its maximum diameter and maximum height in a single spurt of growth, typically in a period of 80-110 days after emergence from the ground. The phrase that bamboo is “one of the fastest growing plants” is attributed to the speed of culm growth. The culm is tubular and hollow with joints or nodes separating the internodes. Nodes may bear small aerial roots or thorns. Internodes of the culm are marked by a sheath scar – a line indicating where a sheath is or was attached to the culm.

Leaf: Four different types of leaf structures are produced by bamboos; namely i) rhizome scales, ii) culm sheath iii) heterophylls and iv) foliar leaves. The foliage leaves have stalked blades and are borne on the branches. These are fully expanded, green in colour and in some species used as fodder in winter months. Leaf sheath is present below leaves and may bear ligules, auricles etc. Some bamboo species are evergreen and some are deciduous (Franklin, 2005). Heterophylls are intermediate between culm sheath and foliar leaves. The number of heterophylls is characteristic of the species.

Flowers: Bamboos are famous for their peculiar flowering behaviour which still remains an enigma and as yet little understood phenomena. Most bamboos flower and set seed at the end of long vegetative growth phase which ranges between 30 to 120 years or more (Janzen, 1976). After flowering and seeding they normally die *en masse*. Flowering in bamboos is

thought to be under genetic control, occurring somewhat like an alarm clock, going off at a preset time (John and Nadgauda, 1999).

Fruit and Seed: Fruit is an indehiscent, one seeded structure known as caryopsis which is typical of family Poaceae (Gopal and Mohan Ram, 1987). Seed set is very poor due to sporadic flowering and low availability because of consumption of large amount of seeds by wild animals especially rodents. Moreover, seeds have low viability, poor storage characteristics and inborn microbial infestation.

Taxonomy of Bamboos

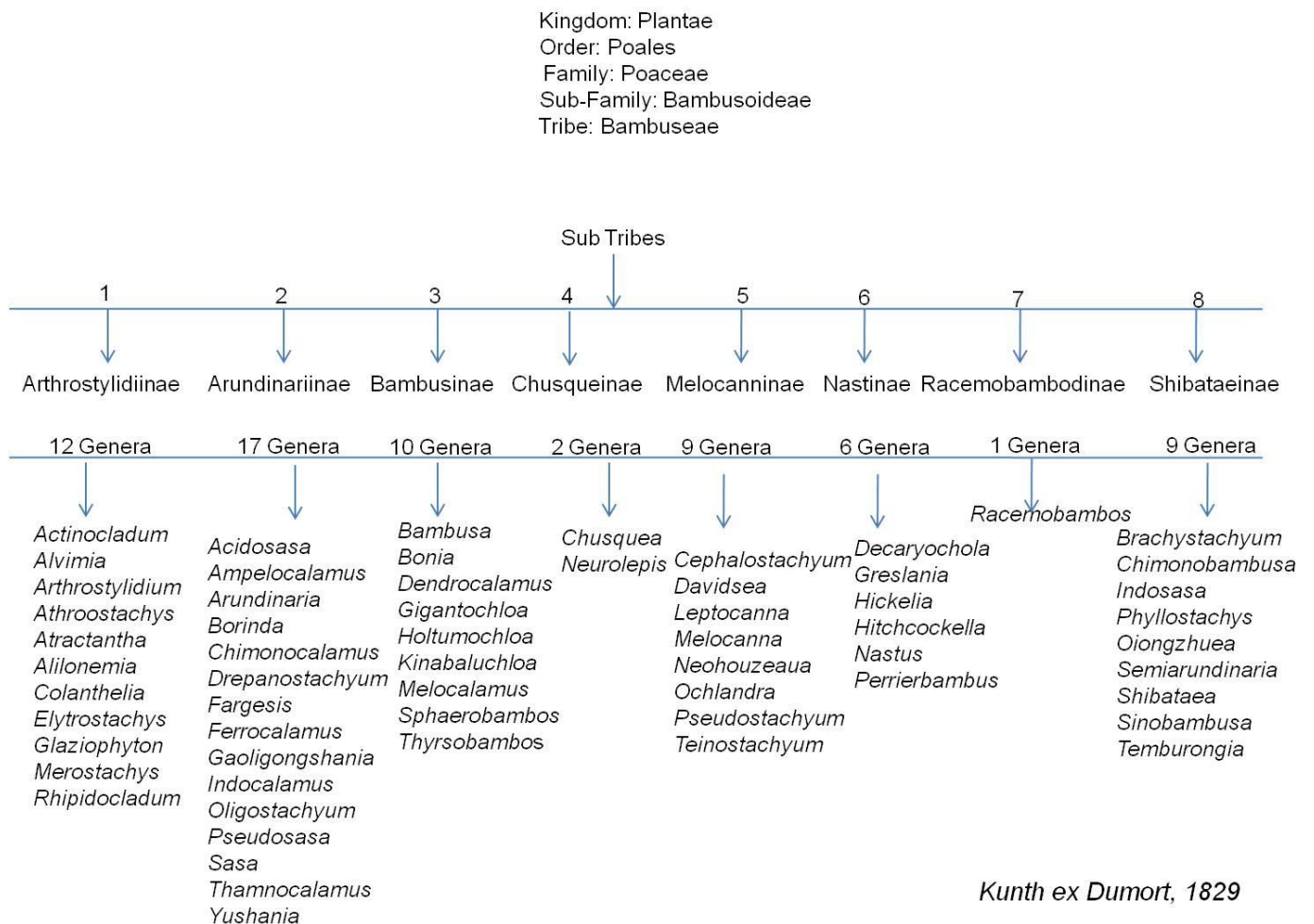


Figure 1.2 Taxonomical position and classification of bamboos.

1.3 Bamboo and Plant Tissue Culture

In the last few decades, the population growth and rapidly increasing bamboo based industries have led to the over exploitation of bamboo resources resulting in severe loss of their natural strands. Other factors responsible for the rapid depletion of bamboo resources are lack of scientific management of existing strands and inadequate efforts to replenish natural bamboo forests and raise new plantations. Over exploitation and poor management practices have caused a rapid depletion of this valuable resource resulting in a wide gap between demand and supply.

Bamboos are conventionally propagated through seeds and vegetative propagation through offsets, rhizomes and culm cuttings. But, both these methods are beset with many problems which restrict their large scale multiplication. Flowering at long intervals and poor seed viability makes their propagation through seeds and genetic improvement by hybridization very difficult. Moreover, there is a lot of heterogeneity in seedling population as bamboos are cross pollinated. The *en masse* death of bamboos results in considerable loss to forest agencies and private cultivators and also has serious ecological consequences (John and Nadguada, 1999).

Likewise, traditional method of vegetative propagation fails to meet the ever increasing demand of bamboos for industrial and commercial applications. Traditional method of vegetative propagation by culm cuttings, offsets and rhizomes limits the number of propagules and again it is slow, cumbersome and labour intensive procedure and hence, inadequate for large scale establishment of bamboo plantations. Another drawback of this method is that the daughter clumps are prone to flowering at the same time as the parent clump (Rao and Rao, 1988). Further due to seasonal specificity of bamboos year round, vegetative propagation becomes difficult (Saxena and Bhojwani, 1993). Because of these reasons, the commercial production of bamboos is uncommon although vast tracts of vacant lands are otherwise available for bamboo cultivation. Therefore, it is imperative to adopt alternative methods of propagation having high multiplication rates to produce large number of plants of improved quality and shortened rotation cycles. In this regard, *in vitro* propagation or micropropagation has emerged as a promising technique for mass propagation of elite bamboos and can lead to the production of healthy, disease free plants and the multiplication can continue throughout the year irrespective of season. Stocks of germplasm can be maintained for many years and it also facilitates the international exchange of germplasm without the inherent risk of spreading pests and pathogens (Sun et al. 2008).

1.4 Status of Bamboo Cultivation in India

India is rich in bamboo genetic resource having 125 indigenous and 10 exotic species spread over an area of 8.96 million hectares which constitutes 12.8% of total forest area (Anonymous, 2003 and Rathore et al. 2009). North-eastern states are rich in bamboo bio-resources and represent about 66% of growing stock of bamboo in the country (Rai and Chauhan, 1998). In India, productivity of bamboo forest is far below (0.3 ton/ha/yr) than their potential and the domestic market of bamboo is worth Rs. 2,043 crores, which could grow upto 2,600 crores by 2015 (Tewari, 1992). Demand of bamboo is estimated to be 26.6 million tones and supply is only 13.47 million tons/yr (Anonymous, 2003). New uses of bamboo, particularly as a substitute of wood, housing sector and value added products will increase further demand of bamboo (Pandey, 2008).

National Mission on Bamboo Applications (NMBA), National Bamboo Mission (NBM) and Department of Biotechnology (DBT), Govt. of India are promoting cultivation and improvement of 15 industrially important species in the country (Swarup and Gambhir, 2008). Planning Commission, Government of India has envisaged 6.0 million bamboo plantations during 10th and 11th five-year plan to meet the growing demand, development of bamboo bio- resource and poverty alleviation. To tap the vast economic potential of bamboos, many organizations in India are working on the propagation of bamboos including Arid Forest Research Institute (AFRI), Tata Energy Research Institute (TERI), Kerala Forest Research Institute (KFRI), Institute of Himalayan Bioresource Technology (IHBT), Technology Information, Forecasting and Assessment Council (TIFAC), Forest Research Institute (FRI), Delhi University, National Chemicals Laboratory (NCL) etc. TIFAC supported by the Planning Commission has been vested with the responsibility of giving a new impetus and direction to bamboo sector in India. National Mission on Bamboo Application (NMBA) is supporting the development of establishment of industrial scale processing units at Jorhat, Assam and Dimapur. In India, nearly 20 lakhs bamboo artisans depend upon the rural agro-industries for their livelihood which will be fulfilled if the bamboo material is made available to them. Hence, Council for Advancement of People's Action and Rural Technology (CAPART) also gives support to voluntary organizations for promoting cultivation of bamboos in various parts of country especially in north Bihar, eastern UP, MP, districts of Orissa and West Bengal. In Tripura, bamboo has been listed as a priority plant by the state forest department and hence need conservation. Still a lot many aspects on bamboo are left unexplored and offer a great scope for research and development. Establishment of bamboo based sector will help overcome the biggest impediment of

irregular and scant supply of bamboo for entrepreneurial use. By successful micropropagation and scientific management, elite bamboo species could be multiplied according to the commercial need. The ecological benefits of these bamboo plantations will help fight global warming and climate change.

1.5 Species Under Consideration

1.5.1. *Dendrocalamus membranaceus*

It has been listed among 18 additional taxa under 'Important' category and is fast becoming a high value crop (www.unep-wcmc.org; www.inbar.int). Major threats to this species include logging of the rainforests in which this bamboo resides, unsustainable land use such as slash-and-burn and shifting agriculture and harvesting for international exportation. It is widely distributed in China, Myanmar and Thailand found in dry/moist tropical and sub-tropical broadleaf forests, montane rain forests and semi-evergreen rain forests. Distribution of *D. membranaceus* in Asia and Pacific is shown in [Figure 1.5.1](#). It grows at an altitudes ranging between 50-3,000 m and due to its large root system, it prevents soil erosion. Besides being an edible bamboo, it is widely used for construction purposes and in paper and pulp industry. After studying 27 bamboos for the edibility of shoots, this bamboo was considered to be excellent from processing point of view. The quality of shoots is acrid when raw. They require pretreatment for 10 minutes in boiling water twice before consumption. According to Chongtham et al. (2011), the edibility of shoots of this bamboo is due to the presence of carbohydrates 5.40; proteins 3.38; amino acids 3.46; ash 0.63; dietary fibers 2.91; fat 0.43; starch 0.23; vitamin C 1.58 and vitamin E 0.65 g/100 g fr.wt. In China, it is used for making chopsticks, shreds and paper. Flowering is sporadic and the flowering cycle varies from 70-90 years. This species was also listed as an important species by Williams and Rao, (1994) for the IPGRI (International Plant Genetic Resource Institute) and was subsequently the focus of international conservation efforts. Therefore, devising a protocol for its propagation by tissue culture is highly desirable.

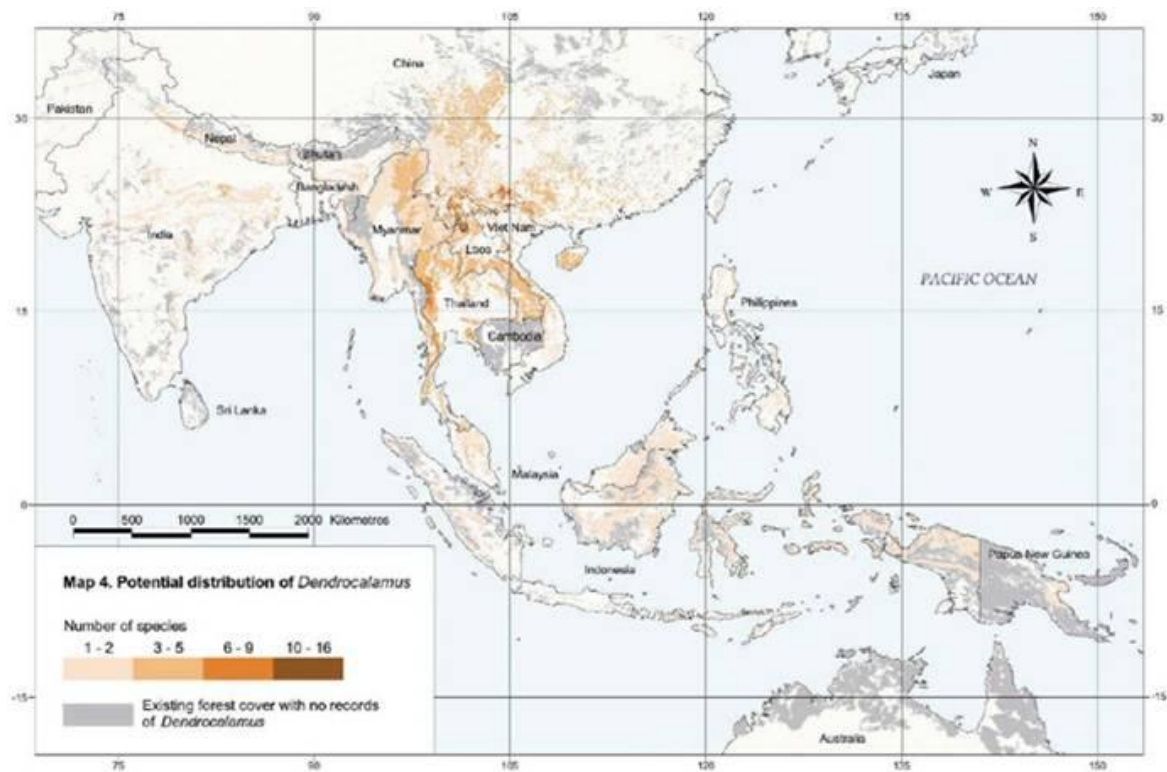


Figure 1.5.1 Distribution of *Dendrocalamus* species in Asia and Pacific
(Source: www.guadubamboo.com/dendrocalamus.html).

1.5.2 *Bambusa balcooa*

Bambusa balcooa Roxb. is accredited as one of the priority bamboo by the National Bamboo Mission (Ministry of Agriculture, Government of India and Rao et al. 1998). It is a multipurpose bamboo species found growing in China, Australia and north-western and north-central Pacific regions. Distribution of *B. balcooa* in Asia and Pacific is shown in [Figure 1.5.2](#). The species is found upto an altitude of 700 m growing in any type of soil but prefers heavy textured soils with good drainage. In India it is distributed in Nagaland, Tripura, Meghalaya, Assam, West Bengal and Bihar extending to Eastern Uttar Pradesh. *B. balcooa* is generally preferred for industrial use due to its rapid growth, excellent flexibility and tensile strength (Bhattacharya et al. 2010). It is quite popular with Vietnamese as food. Among 25 edible species of bamboos tested, protein content was determined to be highest in *B. balcooa* (Bhat et al. 2005). Phytosterols, present in the fermented succulent shoots of *B. balcooa* are the precursors of many pharmaceutically active steroids (Sarangthem and Singh, 2003). Besides, it is the best and strongest species for building purpose and is much used for scaffolding (Tewari, 1992). Other uses include paper pulp, handicrafts, products of wood chip industry, basketry and mats (Ohrnberger, 1999). Gregarious flowering is reported in this

species after 55-60 years and the clump dies immediately after flowering without setting any seeds. Hence, propagation by seeds is a limitation and devising a method for *in vitro* propagation holds greater importance

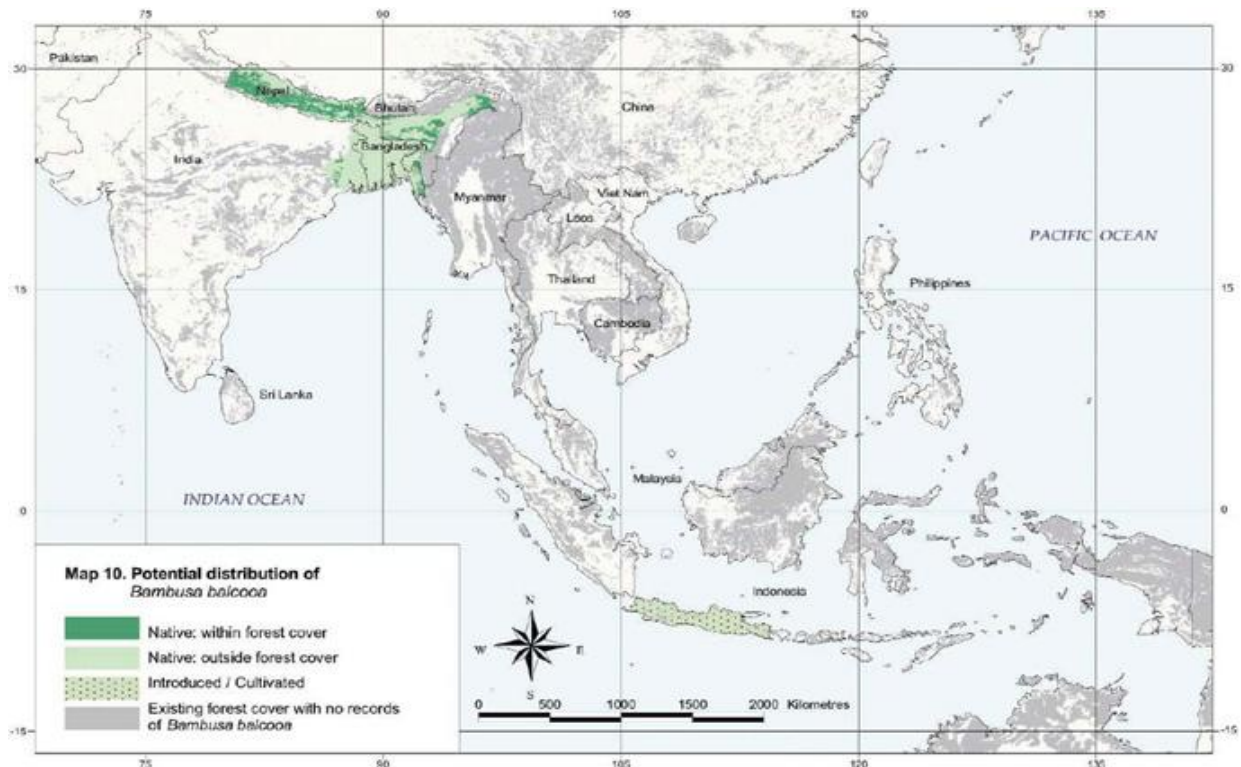


Figure 1.5.2. Distribution of *Bambusa balcooa* in Asia and Pacific (Source: www.guaduabamboo.com).

1.5.3 *Bambusa bambos*

Bambusa bambos called as ‘Giant Thorny’ is listed by IUCN as a priority species along with other endangered species. It is found in Southeast Asian countries and is found almost throughout India and attains best growth in moist deciduous forests upto an altitude of 1000 m. Potential distribution of *B. bambos* in Asia and Pacific is shown in Figure 1.5.3. It prefers rich and moist soil and thrives near perennial rivers and valleys. It grows in dense clumps of closely packed culms. It is used as a raw material for paper and pulp to make panel products, handicrafts and for thatching and roofing. The spiny branches are used for fencing. The leaves are used as fodder and yield an aryurvedic medicine ‘Banslochan’ which is rich in calcium and is used as antipyretic, antispasmodic, antiparalytic, restorative and in debilitating diseases. Leaves also contain digestible crude protein rich in lysine and methionine. The roots show the presence of cynogenic glycosides and this is identical to taxiphylline. Six phenolic acids viz., chlorogenic acid, ferulic acid, coumeric acid, protocatechuic acid, vanillic acid and caffeic acid were identified in the aqueous extract of matured leaves. The phenolic acids

might be responsible for allelopathic (weedicidal) effects (Sanjita et al. 2012). This bamboo is good for boundary planting, riverbank stabilization and soil and water conservation. Young shoots are edible and boiling of shoots in water is not required as a pretreatment before consumption. The shoots are free of acidity, easy to handle, good and tasty when cooked. This bamboo species flowers gregariously after every 40-60 years.

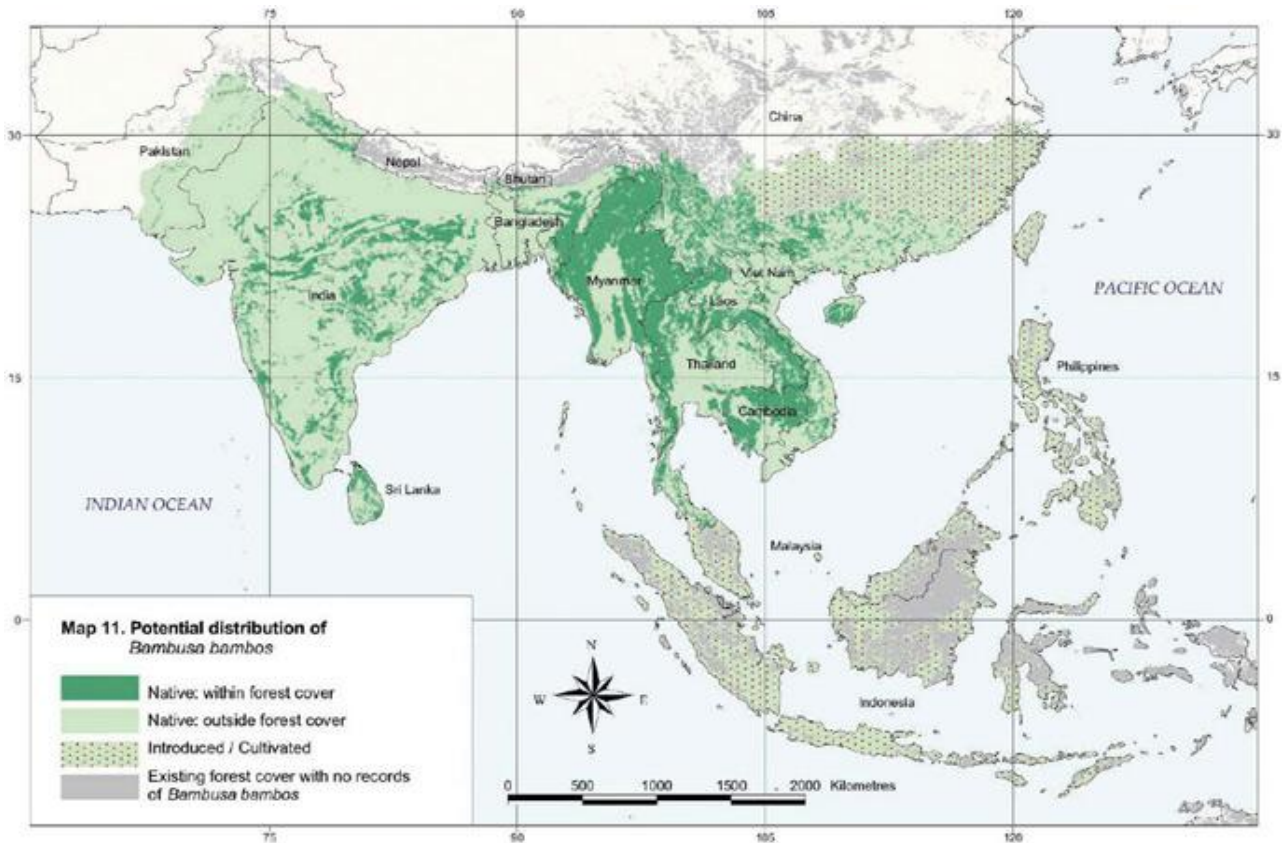


Figure 1.5.3. Potential distribution of *Bambusa bambos* in Asia and Pacific (Source: www.guadubamboo.com/bambusa-bambos.html).

1.6 Rationale and Objectives of the Problem

In view of increasing anthropogenic activities and rapidly eroding natural ecosystem, the natural habitats for a great number of bamboo species are dwindling. Over exploitation of natural bamboo cover coupled with growing demands by rising population and inefficient conventional propagation methods have led to the rapid depletion of natural strands, hence, generating a grave concern about conservation as well as to develop efficient propagation methodologies for large scale propagation of bamboos. With renewed interest in bamboo propagation in India through Bamboo Mission Programmes, the emphasis is on producing quality bamboo material on large scale. *In vitro* conservation and sustainable management of

bamboos should be a high priority as they play an important role in local economies besides having innumerable uses.

All the three species under consideration viz. *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos* have been listed by International Union for Conservation of Nature (IUCN) under the category requiring conservation. In *D. membranaceus* conventional propagation methods cannot produce the plants in large numbers. Topographic position has strong effect on the growth and multiplication of this bamboo. The long flowering cycle of 70 to 90 years produces seeds which are short lived. Culms die after flowering but root system persists from which new plants emerge but this method of propagation is very slow. Propagation by clumps and offsets also proves to be highly cumbersome method. Due to a great demand for planting stock, research must be extended to the use of other parts of *Dendrocalamus* tree such as branches and stem with dormant buds for use as planting material.

Bambusa balcooa has a long flowering cycle of 55-60 years, after which the clump dies without setting seeds. Hence, it can be propagated only through asexual reproduction. Vegetative reproduction through culm cuttings, branch cuttings or rhizome is difficult on account of few and bulky propagules, seasonal specificity and low rooting ability of the cuttings (Tewari, 1992 and Negi and Saxena, 2011a).

Similarly in *B. bambos*, a long flowering cycle of 40-60 years is reported which inhibits its propagation on large scale. Moreover, it being a thorny bamboo, propagation by offsets, culm cuttings and clumps is beset with lots of problems in handling.

Therefore, the conventional propagation techniques are insufficient in all the three species under consideration for the large scale propagation of high quality planting material. In this regard, an efficient *in vitro* propagation technique serves as a viable alternative for large scale establishment of bamboo cover. Hence, developing micropropagation protocol holds great significance in these important edible bamboo species.

Although a few protocols for micropropagation of bamboo species under study from nodal explants are already available, but the present study is relevant in terms of new information on physiological and molecular aspects of *in vitro* raised and the acclimatized plants. The aim of the present study is to investigate the potential of axillary buds and seeds for micropropagation and to make significant improvement in rooting of microshoots in difficult to root species and acclimatization processes. A comparative account of physiological characters of *in vitro* and *ex vitro* grown plants viz. relative water content

(RWC), electrolyte leakage (EL) and chlorophyll content and finally checking the clonal fidelity of *in vitro* raised and acclimatized plants by use of molecular markers are some of the other parameters to be worked upon. Micropropagation from ageing seeds, role of temperature and GA₃ treatments for optimum germination % of seeds in *D. membranaceus* will be worked out. An assessment of clonal fidelity using many Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) as molecular markers will be carried out to ascertain the genetic stability of tissue culture (TC) raised plants.

Objectives:

- 1) To develop and standardize an efficient, reliable and reproducible protocol for mass propagation of *Bambusa bambos*, *Bambusa balcooa* and *Dendrocalamus membranaceus* under *in vitro* conditions.
- 2) Checking their clonal fidelity by RAPD techniques to ensure quality and to study the field performance of the *in vitro* raised plants.

2. Review of Literature

2.1 Micropropagation of Bamboos

Bamboos are considered to be one of the most important renewable forestry species of many Southeast Asian countries where they form integral part of rural economy. There is an increasing need being felt worldwide to enhance the productivity of bamboos due to their multiple uses in order to sustain profitable industries for timber, food, medicine etc. Approximately 1500 commercial applications of bamboo have been identified which include construction and reinforcing fibers, paper, textiles and board, food, combustion and other bioenergy applications, carbon sequestering activity and environment management etc. Bamboo housing is gaining popularity, especially in areas prone to disturbance by earthquakes. Estimates regarding future use of bamboos also indicate that there will be a huge shortage of bamboo planting material in long terms (Subramanian, 1995).

The classical techniques suffer from serious drawbacks and are largely insufficient and inefficient for large scale propagation of bamboos. Conventional propagation of bamboos through seeds cannot be practiced as a reliable method of propagation as sporadic flowering that takes place annually in an isolated clump rarely sets seeds (Ramanayake and Yakandawala, 1998). The vegetative propagation by offset and culm cuttings has proved to be of limited value and is useful for small scale production of clonal planting material. For mass scale propagation (>5,00,000 plants/yr), classical techniques are largely insufficient and tissue culture is the only reliable method (Gielis et al. 2002). Micropropagation can be used as an effective supplementation to conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication to get healthy, disease free and genetically uniform planting material.

Although tissue culture studies have been extensively carried out with other grasses, especially the cereals, bamboos have received rather scant attention (Huang and Murashige, 1983). Although initial reports on regeneration of bamboo plantlets through embryo culture appeared in the late 60's (Alexander and Rao, 1968), advances in tissue culture studies on bamboos is fairly recent. The complete protocol on bamboo micropropagation by Mehta et al. (1982) using seeds of *Bambusa arundinacea* laid the foundation for bamboo micropropagation and *in vitro* conservation. In the recent years, research regarding micropropagation of bamboos has gained impetus (Paranjothy et al. 1990; Saxena, 1990; Sood et al. 1992; Bag et al. 2000; Arya et al. 2002 and Agnihotri et al. 2009) and micropropagation protocols for many bamboo species have been successfully worked out

employing both seed/seedling and mature explants. Unfortunately, very limited success has been achieved with adult tissues because of several technical problems such as seasonal constraints in initiating the cultures, microbial contamination, poor multiplication rates and low rooting frequency. However, multiplication of superior bamboo clumps with desirable traits is possible only with the use of adult tissues than with seed/seedling explants.

The present review is intended to consolidate significant advances made to date in the propagation of bamboos (Table 2.1) and some significant research outcomes are being reviewed in the following pages:

2.1.1 Initiation of Aseptic Cultures

2.1.1.1 Choice of Explant

The choice of starting material is of great significance for the initiation of cultures in bamboo (Gielis, 1999). For carrying out *in vitro* propagation, different explants like seeds, nodal segments, internodal segments, inflorescence, leaf bases etc. have been employed by different workers but nodal explants and seeds are the most commonly used (Rout and Das, 1994 and Negi and Saxena, 2011a). After the first report of micropropagation in *D. strictus* using seeds by Alexander and Rao, (1968), many authors have successfully established the micropropagation protocols using seeds/seedlings explants in different bamboo species like *Dendrocalamus hamiltonii* (Muralidharan, 1995 and Arya et al. 2012); *D. strictus* (Reddy, 2006); *D. giganteus* (Devi et al. 2012); *Bambusa oldhamii* (Thiruvengadam et al. 2011) and in *B. bambos* (Vamil et al. 2010). The use of seeds/seedling explants have many disadvantages such as: 1) insufficient or no knowledge of genetic background 2) restricted availability of seeds for most species and rapid loss of germination capacity and 3) lack of evaluation and comparison of *in vitro* to *in vivo* performance. In addition there is a huge variability in the seed responsiveness in tissue culture (Saxena and Dhawan, 1999). Nodal explants have been the main choice as explants for initiating the cultures as they are least susceptible to genetic modifications. Nodal segments are also preferred due to their easy availability as compared to seeds. Despite a number of impediments associated with the use of adult tissue, successful micropropagation protocols using nodal explants from mature plants have been established in a number of bamboo species viz. *Bambusa balcooa* (Das and Pal, 2005a; Islam and Rahman, 2005; Mudoj and Borathakur, 2009; Negi and Saxena 2011 a and Brar et al. 2014); *B. edulis* (Lin et al. 1998); *B. glaucescens* (Banik, 1987 and Shirin and Rana, 2007); *B. vulgaris* (Nadgir et al. 1984; Gielis, 1999; Ramanayake et al. 2006 and Ndiaye et al. 2006); *B. tulda* (Das and Pal, 2005b; Mishra et al. 2007); *Dendrocalamus giganteus* (Ramanayake et al. 1997); *D. hamiltonii* (Sood et al. 2002a; Agnihotri and Nandi,

2009 and Agnihotri et al. 2009); *D. strictus* (Chaturvedi et al. 1993 and Ravikumar et al. 1998); *D. asper* (Singh et al. 2012 b and Nadha et al. 2013); *Guadua angustifolia* (Rathore et al. 2009 and Nadha et al. 2011) and many more. Some researchers have also used inflorescence explants for establishing protocols for shoot proliferation in *D. giganteus* (Ramanayake, 1998); *D. latiflorus* (Lin et al. 2006); *D. asper* (Arya et al. 2008 a) and *Bambusa edulis* (Lin et al. 2005). Other reports include use of mature anthers in *Sinocalamus latiflorus* (Tsay et al. 1990); caryopses in *B. bambos* (Kapoor and Rao, 2006); pseudospikelets in *B. balcooa* (Gillis et al. 2007); roots in *B. beecheyana* (Yeh and Chang, 1986 b); pollen grains in *D. strictus* (Nadgauda et al. 1993) and internodal segments of *Dendrocalamus asper* (Shrotri et al. 2012).

2.1.1.2 Effect of Season

The effect of a particular season and climatic factors on bud break frequency has been worked out by many workers who reported a correlation of season with the growth of the plant. In *B. balcooa*, Negi and Saxena, (2011a) reported 100% bud break when the cultures were initiated in the month of July, whereas Das and Pal, (2005a) observed lowest morphogenetic competence of the axillary buds from January to May and highest from June to October. Anand et al. (2013) observed spring (February and March) to be the best period for raising aseptic cultures in *B. bambos* when 93% bud break was achieved. In *Arundinacea callosa*, Devi and Sharma, (2009) reported the successful initiation of aseptic cultures and highest frequency of bud-break when explants were collected after the monsoon when bacterial contamination was lowest. In *Dendrocalamus asper*, Nadha et al. (2013) reported the months of January and February to be the best for initiating aseptic cultures and to get maximum bud break.

2.1.1.3 Effect of Sterilants

Failure of surface sterilization procedures to produce aseptic cultures is a main problem in bamboos and play a major role in initiation of cultures. In bamboos, the seeds require more severe surface sterilization treatment than nodal explants taken from mature culms due to persistent microbial contamination as also observed by Duad et al. (2012) in *Aquilaria malaccensis*. Different strategies have been employed by different workers to counter/eliminate microbial contamination. Sodium hypochlorite (NaOCl) has been used as an effective sterilant at a concentration of 10% by Mukunthakumar et al. (1999) in *Dendrocalamus brandisii*, 4% by Godbole et al. (2003) in *D. hamiltonii* and 30% by Woods et al. (1992) in *Otatea acuminata*. Similarly, mercuric chloride (HgCl₂) was used at different concentrations of 0.2% for 25 minutes in *Bambusa vulgaris*, *Dendrocalamus strictus* and *D.*

giganteus (Rout and Das, 1994); 0.04% for 6 minutes in *D. hamiltonii* (Sood et al. 1992) and *Bambusa nutans* (Mehta et al. 2010). Arya and Sharma, (1998) used 5% cetavelon solution in *B. bambos* and ethanol at a concentration of 70% was used by Rathore et al. (2009) in *D. stocksii*. Ali et al. (2009) reported the use of antibiotics rifampicin (0.1%), streptomycin (0.1%) and ciprofloxacin (0.25%) in *D. asper*. Nadha et al. (2012) developed a strategy to identify and inhibit intriguing microbial contamination in *Guadua angustifolia* cultures by using 10 µg/ml of kanamycin alongwith 10 to 15 µg/ml of streptomycin sulphate. Disinfection procedure for seeds of some bamboo species have been compiled in [Table 2.1.1.3](#).

Table 2.1.1.3 Disinfection procedure for seeds of some bamboo species

Species	Disinfection Protocol	Reference
<i>Dendrocalamus strictus</i>	Kept the dehusked seeds in running water for 1 hr, then dipped in 5% teepol for 5 min. and washed in running tap water and distilled water; washed seeds with rectified spirit for few seconds and sterilized with 0.1% HgCl ₂ for 15 min.	Niraula and Bhandary, (1987)
	Washed seeds with 2% teepol solution on a magnetic stirrer for 5 min. and in running tap water for 15-20 min. Rinsed in distilled water and immersed in chlorine water and washed thoroughly in sterile distilled water.	Rao et al. (1985) and Rao et al. (1987)
<i>Dendrocalamus membranaceus</i>	Surface-sterilized with 75% ethanol for 1 min. and dipped into 10% NaOCl for 15 min. and, then rinsed thoroughly in sterile distilled water.	Vongvijitra, (1988)
	Surface sterilized seeds with 0.1% HgCl ₂ and 15% bleach for 10 minutes each was effective in raising aseptic cultures.	Brar et al. (2013)
<i>Oatea acuminata</i> <i>O. aztecorum</i>	Washed for 10 min. in water containing a few drops of detergent; rinsed in distilled water; dipped in 70% ethanol for 1 min.; immersed for 15 min. in 30% commercial bleach and rinsed 3 times in sterile water. Finally rinsed with sterile water at a pH of 3.5.	Woods et al. (1992)
<i>Sinocalamus latiflorus</i>	Sterilized with 75% ethanol for 1 min. and dipped into 2% NaOCl for 10 min. and rinsed thoroughly in autoclaved distilled water.	Yeh and Chang, (1987)
54 species from 15 genera*	Sprayed with 70% ethanol and surface-sterilized for 30 min. in 1% NaOCl and washed 3 times in sterile water.	Prutpongse and Gavinlertvatana, (1992)

**Arundinaria pusila*, *A. superecta*, *Atatea aztecorum*, *Bambusa arundinacea*, *B. flexuosa*, *B. glaucescens*, *B. gracilis*, *B. humilis*, *B. longispiculata*, *B. multiplex (variegata)*, *B. nigra*, *B. multiplex*, *B. oldhamii*, *B. polymorpha*, *B. ventricosa*, *B. ventricosa (variegata)*, *B. vulgaris*, *B. cv. Dam Khan*, *B. cv. Bong ban*, *B. Bong Naew*, *Cephalostachyum pergracile*, *Dendrocalamus asper*, *D. giganteus*, *D. hamiltonii*, *D. Bong Kaiy*, *D. latiflorus*, *D. membranaceus*, *D. nutans*, *D. strictus*, *Dinochloa scandens*, *Gigantochloa albociliata*, *G. apus*, *G. auricurata*, *G. compressa*, *G. densa*, *G. hasskarliana*, *G. hossuesii*, *Hibanobambusa triangullans*, *Helocatamus compactiflorus*, *Oxytenanthera albociliata*, *Phyllostachys aurea*, *P. bambusoides*, *P. gramineus*, *P. nigra f. megurochiku*, *P. pubescens*, *P. sulphurea*, *P. viridis*, *P. iaponica*, *Sasa fortunei*, *Sasaella sumekoana*, *Schizostachyum aciculare*, *S. brachycladum*, *S. zollingeri*, *Semiarundinaria fastuosa*, and *Thyrsostachys oliveri*

2.1.1.4 Choice of Medium

For initiation of cultures from seeds and nodal explants, various media were employed by different workers. A perusal of the literature depicts that MS medium is the most widely used medium for inducing bud break in nodal explants as reported in *Dendrocalamus hamiltonii* (Sood et al. 1992), *D. giganteus* (Ramanayake and Yakandawala, 1997); *D. latiflorus* (Lin et al. 2007 b); *D. asper* (Arya et al. 2008 and Nadha et al. 2013); *D. hamiltonii* (Agnihotri et al. 2009); *Bambusa vulgaris* (Rout and Das, 1997); *B. edulis* (Lin and Chang, 1998) and *B. balcooa* (Das and Pal, 2005 a; Negi and Saxena, 2011a and Brar et al. 2014) etc. For the germination of bamboo seeds, White's medium (White, 1963) was recommended by some researchers (Alexander and Rao, 1968; Nadgir et al. 1984; Nadgauda et al. 1997 b and Ravikumar et al. 1998). In *Dendrocalamus asper*, Arya et al. (1999) used Woody Plant Medium for germination of seeds. Ndiaye et al. (2006) tested four basal media viz. MS medium, Gamborg's medium, Lloyd and Crown medium (1981) and modified MS medium for evaluating regeneration efficiency of nodal explants in *B. vulgaris* and observed that modified MS medium showed highest rate of regeneration of 100%. Even with MS medium, different strengths have been employed by many researchers for inducing bud break. Sood et al. (1992) observed sprouting of nodal explants of *D. hamiltonii* on half strength MS medium within 10 days. Similarly, Roohi et al. (1991) and Yasodha et al. (2010) also preferred ½ MS medium for better shoot proliferation in *B. balcooa* and *D. giganteus* respectively.

2.1.2 Axillary Shoot Proliferation

2.1.2.1 Role of Plant Growth Regulators (PGRs)

Cytokinins help in overcoming the apical dominance in the growth of axillary buds from mature explants in bamboos. In general, cytokinins promote shoot formation and BAP has been the most commonly used cytokinin for effective axillary bud proliferation. Depending upon the species the growth hormones can be used at low to high levels. Mudoj and Borthakur, (2009) used 1 mg/l of BAP for continuous shoot proliferation in *Bambusa balcooa* by sub-culturing 2-3 shoots/cluster. In *B. oldhamii*, Thiruvengadam et al. (2011) reported the use of 4.4 µM of BAP for axillary shoot proliferation from mature nodal explants. Shroti et al. (2012) reported a multiplication rate of 15-16 folds in *Dendrocalamus asper* when MS medium was supplemented with 2.0 mg/l BA. Arya et al. (2009) used 4.4 µM of BA for achieving maximum shoot multiplication rate and for better overall growth of cultures in *D. hamiltonii*. It was used at high concentration of 5 mg/l in *Guadua angustifolia* as reported by Jimenez et al. (2006) and 6-12 mg/l BAP was used for continuously proliferating axillary

shoots in *Dendrocalamus giganteus* (Ramanayake and Yakandawala, 1997). For high frequency multiple shoot induction, a combination of two cytokinins has also been used by many workers as reported in *Bambusa wamin*, (Arshad et al. 2005); *B. glaucescens*, (Shirin and Rana 2007); *B. bambos*, (Anand et al. 2013) and in *Ochlandra wightii*, (Bejoy et al. 2012). Inclusion of low concentrations of auxins in the culture medium alongwith the cytokinins promoted a higher rate of shoot multiplication in many cases. In *Dendrocalamus hamiltonii*, Agnihotri and Nandi, (2009) reported enhanced shoot proliferation on medium supplemented with 8.0 μM of BAP and 1 μM of NAA. Best shoot multiplication was obtained on MS medium supplemented with 4.4 μM BAP and 0.53 μM NAA in *Bambusa balcooa* (Brar et al. 2013). Diab and Mohamed, (2008) used 2.68 μM of NAA and 4.4 μM of BAP in *Oxyanthera abyssinica* and 5.0 mg/l BA and 0.2 mg/l NAA was reportedly used by Sanjaya et al. (2005) for high frequency shoot multiplication in *Psuedoxyanthera stocksii*. In *Thamnocalamus spathiflorus*, Bag et al. (2000) reported use of 5.0 μM of BAP in conjunction with 1.0 μM of IBA for multiple shoot proliferation.

Administration of cytokinin like TDZ also proved effective for shoot proliferation in many bamboo species. In *Bambusa edulis*, Lin and Chang, (1998) reported the use of 0.1 mg/l TDZ for induction and proliferation of shoots while Singh et al. (2000) used 1.0 mg/l TDZ for *in vitro* shoot multiplication in *Dendrocalamus strictus*. In *B. oldhamii*, the use of 0.45 μM TDZ by Lin et al. (2007a) resulted in proliferation of shoots from nodal explants. In *Arundinaria gigantea*, role of 0.01 μM TDZ in multiple shoot induction has been demonstrated by Baldwin et al. (2009). In *Dendrocalamus asper*, both Singh et al. (2012 b) and Nadha et al. (2013) reported the use of adenine sulphate in conjunction with cytokinin for axillary shoot proliferation.

2.1.2.2 Role of Sucrose

Sucrose (2-3%) is the carbohydrate of choice for establishment and maintenance of *in vitro* cultures of most plant species. Mostly workers have reported the use of 3% sucrose as originally used by Murashige and Skoog's, (1962) but some have used 2% sucrose for the better proliferation of cultures as reported in *Dendrocalamus membranaceus* and *Bambusa nutans* (Yasodha et al. 1997); *Dendrocalamus giganteus* (Ramanayake et al. 2001); *Bambusa bambos* (Kapoor and Rao, 2006) and *B. nutans* (Yasodha et al. 2008) etc. In *B. balcooa*, Brar et al. (2014) reported shoot proliferation in medium supplemented with 1% sucrose forming 19.8 ± 1.4 shoots whereas Roohi et al. (1991) reported the use of 6% sucrose for shoot multiplication. In *Dendrocalamus giganteus*, a high level of sucrose i.e. 4% adversely affected the shoot growth forming only 13.3 shoots compared to 34.3 and 39.2 shoots obtained on 2 and 3%

sucrose respectively (Ramanayake et al. 2001). Hence, the optimum level of exogenous carbohydrate varies with every species and affects the physiology and differentiation of tissues.

2.1.2.3 Role of Other Additives

Inclusion of additives into the culture medium is helpful in eliminating the problems associated with browning of the medium and drying of shoots including recalcitrance induced in the *in vitro* cultures. In *B. glaucescens*, Jullien and Van, (1994) reported the use of 500 mg/l polyvinyl pyrrolidone (PVP) to minimize the effect of phenolic compounds during the proliferation of axillary buds. In *Pseudoxanthera stocksii*, Sanjaya et al. (2005) achieved high frequency multiple shoot formation on MS medium supplemented with ascorbic acid (283.93 μM), citric acid (118.10 μM), cysteine (104.04 μM), and glutamine (342.24 μM) as additives. Banik, (1987) and Dekkers and Rao, (1989) reported the use of activated charcoal in the media to achieve maximum multiple shoot proliferation in *Bambusa glaucescens* and *B. ventricosa* respectively. Addition of 10% coconut milk (CM) for better shoot proliferation was reported in *D. longispathus* by Saxena and Bhojwani, (1993) whereas in *D. giganteus*, Ramanayake and Yakandawala, (1997) reported that the addition of casein hydrolysate (CH) to the medium has the same effect as higher levels of BAP (12 mg/l).

2.1.3 Rooting of Shoots

Rooting is the major problem encountered during *in vitro* multiplication of bamboos. Low rooting frequencies are the major bottleneck to developing commercially viable protocols. Bamboos are known to have varied inherent ability to root both under *in vitro* and *ex vitro* conditions and many factors influence the response of shoots to the rooting media. Different auxins have been tried by different groups and the role of IBA and NAA in root induction is well documented. According to Lin et al. (2007a), only 22% of the multiple shoots rooted on hormone-free semisolid medium in *Bambusa oldhamii* and this percentage increased to 78% after the shoots were transferred to medium containing 26.85 mM of NAA. NAA alone has also been reportedly used for rooting of regenerated shoots in *B. balcooa*, (Roohi et al. 1991, Brar et al. 2014) and *B. bambos* (Arya and Sharma, 1998). Maximum rooting, however, has been achieved in bamboos by the use of IBA as reported by many workers. IBA was used in *B. vulgaris* (Rout and Das, 1997); *B. glaucescens* (Shirin and Rana, 2007); *B. bambos* (Anand et al. 2013); *Dendrocalamus brandisii* (Mukunthakumar et al. 1999); *D. hamiltonii*, (Agnihotri et al. 2009); *D. giganteus* (Devi et al. 2012); *Melocanna baccifera* (Kant et al. 2009) and *Gigantochloa artoviolaceae* (Bisht et al. 2010) and in many more for induction and proliferation of roots. IAA alone has also been used for induction of roots in *Dendrocalamus longispathus* (Saxena and Bhojwani, 1993); *D. farinosus* (Hu et al. 2011)

and *Bambusa nutans* (Negi and Saxena, 2011b). The combination of two auxins (IBA and NAA) was also used in some species like *Dendrocalamus strictus* (Chaturvedi et al. 1993); *D. asper* (Singh et al. 2012 b) and *Bambusa oldhamii* (Thiruvengadam et al. 2011) for better rooting percentage. In *B. balcooa*, Negi and Saxena, (2010) reported the use of three auxins IBA, IAA and NAA together. Although majority of the workers reported the use of auxins for rooting yet a few reports of rooting on hormone free basal MS medium have also been mentioned in literature like in *Dendrocalamus strictus* (Shirgurkar et al. 1996) and *Phyllostachys meyeri* (Ogita et al. 2008) etc. The role of TDZ in rooting has also been highlighted in *Bambusa edulis* (Lin and Chang, 1998); *B. vulgaris* (Ramanayake et al. 2006) and *Dendrocalamus strictus* (Singh et al. 2001).

Additives, auxin protector coumarin and amino acids were required by certain species of bamboos to enhance the *in vitro* rooting. In *Dendrocalamus strictus* (Nadgir et al. 1984); *Bambusa edulis* (Lin et al. 2004 a) and *B. vulgaris* (Ramanayake et al. 2006), coconut milk (0.1 to 10%) was used as an additive for the induction of roots. Activated charcoal is frequently added to absorb root inhibiting agents. The role of coumarin for enhanced rooting has been advocated in *B. balcooa* (Roohi et al. 1991); *Dendrocalamus longispathus* (Saxena and Bhojwani, 1993); *D. giganteus* (Ramanayake and Yakandawala, 1997) and *D. hamiltonii* (Sood et al. 2002 a). Diacamba and Picloram were also tested in *Bambusa oldhamii* by Lin et al. (2007a) for induction of roots with not more than 50% rooting reported. Other phenolic compounds such as caffeic acid and phloroglucinol were also reported to regulate rooting in species that are difficult to root like *Dendrocalamus strictus* as reported by Chaturvedi et al. (1993).

Modified MS medium with major inorganic salts reduced to half or quarter strength have been tried to enhance rooting percentage in bamboos. Half strength MS medium was used in *Dendrocalamus giganteus* (Ramanayake and Yakandawala, 1997); *D. strictus* (Saxena and Dhawan, 1999; Mishra et al. 2001; Singh et al. 2001 and Reddy et al. 2006); *D. asper* (Singh et al. 2012 b and Nadha et al. 2013); *D. hamiltonii* (Bag et al. 2012); *Bambusa vulgaris* (Rout and Das, 1997); *B. bambos* (Anand et al. 2013) and *Melocanna baccifera* (Kant et al. 2009) and many more for rooting. Negi and Saxena, (2010) used ½ MS medium for root induction in *Bambusa balcooa* whereas full strength MS medium gave the best response as demonstrated by Brar et al. (2014). Rathore et al. (2009) used ¼ strength MS medium in *Dendrocalamus stocksii* for best rooting.

2.1.4 Hardening and Acclimatization

Micropropagated plantlets are associated with several physiological and anatomical abnormalities during *in vitro* growth such as low photosynthesis, non-proper functioning of stomata, malfunctioning of water housekeeping systems mainly due to high humidity inside the culture vessel (Kozai, 1991). Therefore, they need to be hardened or acclimatized before their transfer to the *ex vitro* conditions to ensure better survival. Various potting mixtures have been used by different workers for acclimatization of bamboo plantlets. Comparison of different potting mixtures has shown varied response in different species. Soilrite: sand (2:1) was used in *Bambusa bambos* (Arya and Sharma, 1998) while rooted plantlets of *B. edulis* grew well when they were transferred to pots containing non-sterile vermiculite: peat: perlite 1:1:1 (Lin and Chang, 1998). In *Dendrocalamus brandisii*, Mukunthakumar et al. (1999) reported soil: farmyard manure (FYM) (1:1) as the major potting mixture while Singh et al. (2001) achieved maximum growth of *D. strictus* plants on a mixture of sand and garden soil (1:1). Ali et al. (2009) used soil: fine sand: peat moss (1:1:1) for successful acclimatization of plants in *D. asper*. Soil: sand: FYM (1:1:1) was used successfully in *D. hamiltonii* (Godbole et al. 2002 and Arya et al. 2009); *Gigantochloa artoviolaceae* (Bisht et al. 2010) and *Bambusa nutans* (Sharma and Kalia, 2012). Negi and Saxena, (2011 b) tried various potting mixtures in *B. nutans* with varying levels of success. They reported hardening survival of 98% in the soil: agropeat (2:1) mixture, 92% in the soil: agropeat (1:1) mixture, 90% in agropeat mixture, 88% in soil: agropeat mixture (3:1) and 80% in the soil alone. Hence, it was found that survival rate during hardening was dependent upon the type and proper ratio of the potting mixture.

2.1.5 Somatic Embryogenesis

Because of low rooting frequency in bamboos, induction of somatic embryogenesis and germination of embryos into plantlets is a promising alternative. The regeneration through embryogenesis is affected by many factors such as bamboo species (including cultivars, genotypes and ecotypes), type and age of explants, type of basal medium and type and concentration of plant growth regulators (Godbole et al. 2002). The protocols for somatic embryogenesis of bamboos were developed using various explants like seeds (Mehta et al. 1982), shoot tip (Huang et al. 1983 and Hu et al. 2011); young leaf (Vasana 1985 and Jullien and Van, 1995); inflorescence (Yeh and Chang 1986 b and Gillis et al. 2007); anther (Tsay et al 1990) and mature zygotic embryos (Rout and Das, 1994 and Yuan et al. 2013). Regeneration through somatic embryogenesis has been reported in *Dendrocalamus strictus*, *D. giganteus* and *B. vulgaris* (Rout and Das, 1994); *Dendrocalamus strictus* (Saxena and

Dhawan, 1999) and *D. hamiltonii* (Godbole et al. 2002) and in many more species. The role of 2,4-D in the induction of embryogenic calli was demonstrated in *Bambusa bambos* (Mehta et al. 1982); *B. glaucescens* (Jullien and Van, 1994); *Bambusa balcooa* (Gillis et al. 2007). *Sinocalamus latiflorus* (Yeh and Chang, 1987); *Dendrocalamus latiflorus* (Zamora et al. 1988) and *D. giganteus* (Ramanayake and Wanniarachchi, 2003). Another auxin 2,4,5-T (2 to 5 mg/l) was used for induction of embryogenic calli in *D. longispathus* (Saxena and Bhojwani, 1991) and in *D. farinosus* (Hu et al. 2011). The use of an auxin along with cytokinin was also reported for induction of embryogenic callus as reported in *Bambusa oldhamii* (Yeh and Chang 1986 a); *B. beecheyana* (Chang and Lan, 1995); *Dendrocalamus asper* (Shrotri et al. 2012) and *D. hamiltonii* (Bag et al. 2012) and in many more. TDZ (0.45 μ M) was used effectively for embryogenic callus induction in *Bambusa edulis* as reported by Lin et al. (2004a).

Cytokinin BAP has been used for regeneration from somatic embryos in many species including *Dendrocalamus latiflorus* (Zamora et al. 1988); *Bambusa ventricosa* (Cheah and Chaille, 2011) while use of Kinetin was employed for regeneration in few species as in *Dendrocalamus strictus* (Rout and Das, 1994) and *D. giganteus* (Devi et al. 2012) etc. Somatic embryos formed in *D. strictus* multiplied rapidly on medium containing IBA (2×10^{-6} M) as reported by Saxena and Dhawan, (1999) and IBA was also reportedly used by Devi et al. (2012) in *D. giganteus*. Zeatin (5-7 mg/l) was incorporated into MS medium for better regeneration of somatic embryos in moso bamboo as reported by Yuan et al. (2013). Auxins like NAA were also used for regeneration experiments as in *Bambusa edulis* (Lin et al. 2003) for better regeneration.

Half strength MS media was used by many workers for improvement in regeneration ability during somatic embryogenesis as reported in *Bambusa vulgaris* (Rout and Das, 1994); *B. nutans* (Mehta et al. 2010); *Dendrocalamus strictus* (Saxena and Dhawan, 1999) and *Dendrocalamus hamiltonii* (Godbole et al. 2002 and Bag et al. 2012). In *Bambusa nutans*, full strength MS medium favoured regeneration of somatic embryos into plantlets as reported by Mehta et al. (2010) etc. In few bamboo species, N₆, HB, White's and B₅ medium were used for regeneration and embryogenesis besides MS basal medium. Alexander and Rao, (1968) used White's medium for embryo culture in *Dendrocalamus strictus* while N₆ medium was reportedly used in *Bambusa bambos* (Mehta et al. 1982) and *Thyrostachys* spp., (Dekkers et al. 1985). In *Bambusa bambos* and *Dendrocalamus longispathus*, B₅ medium was demonstrated to be the best for somatic embryogenesis (Rao and Rao, 1988 and Saxena and Bhojwani, 1993).

Rao and Rao, (1988) reported germination of mature embryos of *Bambusa bambos* on 2% sucrose which later produced long roots. In *Dendrocalamus giganteus*, Ramanayake and Wanniarachchi, (2003) reportedly used 4% sucrose for conducting their experiments. High sucrose (5 to 8%) favoured somatic embryogenesis in many bamboos by reducing the frequency of albinos and providing impetus to the growing embryoids leading to their germination into plantlets as reported in *D. hamiltonii* (Sood et al. 1994; Godbole et al. 2002); *Bambusa oldhamii* (Yeh and Chang, 1986a) and *B. edulis* (Lin et al. 2004a) and in many more. Glucose (2%) was used for proliferation of somatic embryos in *B. nutans* (Mehta et al. 2010).

Many chemical additives including osmoticums, anti-oxidants, and ethylene inhibitors influence plant regeneration during somatic embryogenesis. In *Bambusa bambos* and *Dendrocalamus strictus*, PVP was added to the medium for reduction of tissue browning, high germination and multiplication of somatic embryos (Mehta et al. 1982 and Saxena and Dhawan, 1999). In *Bambusa glaucescens*, Jullien and Van, (1994) reported the use of 0.6 μM ABA for achieving embryogenesis. Rout and Das, (1994) incorporated 10 mg/l of adenine sulphate alongwith 0.5 mg/l GA_3 in *B. vulgaris*, *Dendrocalamus giganteus* and *D. strictus* for somatic embryogenesis. Lin et al. (2004 a) mentioned the use of 0.1% v/v coconut milk (CM) in *Bambusa edulis*. The use of ascorbic acid (20 mg/l) by Mehta et al. (2010) in *B. nutans* for the maturation of embryos yielded positive results.

2.1.6 Seed Culture

Potential application of tissue culture for propagation using seeds in bamboos is well documented. The major constraints for germination of seeds in bamboo are very long intermast period (Nadgauda et al. 1997 b) and their short viability period (Brar et al. 2013). Control of contamination and establishment of aseptic cultures is a priority requirement for successful germination of seeds. Various procedures for surface sterilization have been developed to counter this problem (Thakur and Sood, 2006). Use of ethanol, commercial bleach and HgCl_2 in raising aseptic cultures is quite a common practice (Huarte and Garcia 2009 and Liu et al. 2011). Bamboo seeds germinate within 3-7 days, lose their viability within 1-2 months (Surendran et al. 2003) and germination potential is season dependent. Bamboo seeds germinate at higher percentage under shade than in direct sunlight. Thus bamboo seeds can be considered as negatively photoblastic (Banik, 1996). Among the abiotic factors, temperature is considered an important issue for seed germination, because it persuades the rate of water absorption and additional substrates necessary for the growth and development (Buriro et al. 2011).

The effects of exogenous application of GA₃ treatments on bamboo seeds was studied earlier by Richa et al. (2006) who found that GA₃ regulates germination in numerous ways. Pretreatment of seeds with 50 mg/l GA₃ significantly enhanced germination % as observed in *D. membranaceus* (Brar et al. 2013). With application of GA₃, several physiological changes occur in seeds and ratio of levels of ABA (growth inhibitor): GA₃ (growth regulator) gets altered triggering germination (Hilhorst and Karssen, 1992). Germination may be dependent upon ability of seeds to utilize reserves more efficiently (Rao and Sinha, 1993). Starch is the major reserve material present in the endosperm of *D. membranaceus* bamboo seeds as demonstrated by starch test (Brar et al. 2013). Seed viability is generally very low in bamboos as endogenous levels of auxins and ABA in seeds is found to be one of the major factors related to the loss in the seed viability in stored bamboo seeds as depicted by Richa et al. (2006). Ageing of seeds is progressive and leads to death of seed and hence non viability (Schmidt, 2007). Guzman et al. (2011) tested the non viability of seeds using tetrazolium chloride (TTC) and was considered important factor in seed trade, crop production and also in germplasm conservation and management.

2.1.7 Evaluation of *In Vitro* Plants and *Ex Vitro* Hardened Plants

Acclimatization of micropropagated plantlets to *ex vitro* conditions is a crucial step to cope with the new environment for better growth and development. The general observation is that leaf area increases under long day conditions of 12 hours light when plants are growing in field as compared to *in vitro* conditions of the culture room. A comparison of *in vitro* raised and *ex vitro* grown plants has been done and valuable information regarding the physiological parameters and stomatal density was depicted in *Thamnocalamus spathiflorus* and *Dendrocalamus hamiltonii* (Bag et al. 2000 and Bag et al. 2012).

Relative water content is widely accepted as a reproducible and meaningful index of plant water status (Weatherly, 1951). Slatyer and Taylor, (1960) reported that water potential measures the energetic status of water inside the leaf cells. Water deficit is very common in the production of most crops and numerous studies have indicated that this deficit can have substantial negative impacts on plant growth and development (Carrow, 1996). Tissue-cultured plantlets behave as hydraulically integrated units, in which there must be a co-ordination between control of water loss by the shoot and uptake of water by the root to maintain a favourable plant water balance as reported by Juan et al. (1995). In bamboos, very few studies have been reported on the water status of plants. Water use efficiency (WUE) was calculated in *Thamnocalamus spathiflorus* and *Dendrocalamus hamiltonii* which are among

the few reports which take into account the rates of photosynthesis and transpiration (Bag et al. 2000 and Bag et al. 2012).

The typical objective of conducting electrolyte leakage studies is to assess injury presumably at the membrane level resulting from an environmental stress (Juan et al. 1995). Leakiness will vary in relation to the membrane ability to take up and retain solutes and therefore, will reflect stress-induced changes in both membrane potentials and membrane permeability (Minorsky et al. 1985). In plant membranes, these changes are often associated with increase in permeability and loss of integrity (Campos et al. 2003). According to the studies of Murphy and Alvin, (1997) unstressed and undamaged plant cells maintain electrolytes within the membrane.

Bamboo lignin is composed of guaiacyl, syringyl and p-hydroxy phenylpropane units and also contain 5-10% p-coumaric acid ester as depicted by the work of Higuchi, (1987). The members of Poaceae have hydroxy cinnamic acid moieties which cross links with cell wall polysaccharides and participate with lignin to generate polysaccharide lignin complexes which lead to an increase in cell wall rigidity as reported by Morrison et al. (1998). In *Phyllostachys* spp., Lin et al. (2002) have demonstrated that lignification of bamboos increases with ageing. On the other hand, Lybeer and Koch, (2005) studied lignin distribution and did not observe any difference in lignin content of *Gigantochloa levis* between the various ages and between flowering and non-flowering culms.

The reports available on chlorophyll content in bamboos are very less. Low photosynthetic rates for the plants *in vitro* have been reported by Grout, (1988) which leads to low chlorophyll content in bamboos. The possible reason may be due to less CO₂ and low irradiance under *in vitro* conditions. In *Bambusa balcooa*, Brar et al. (2014) also reported higher values of chlorophyll and carotenoids under *ex vitro* conditions due to higher levels of CO₂ and light intensities. Acetone was used as the solvent of choice for extraction of chlorophyll in maximum studies as reported earlier by Chang et al. (1998) and Wu et al. (2002), DMSO has been used by Hiscox and Israelstam, (1979) and DMF by Moran and Porath, (1980). According to Wu et al. (2002) extraction efficiencies of solvents may be attributed due to different stabilities of solvents. For extraction of chlorophyll from bamboo culms in *Phyllostachys pubescens*, ultrasonics has been reported to be better method than the traditional method of grinding and centrifugation (Chang et al. 1998 and Wu et al. 2002). Malenovsky et al. (2006) reported that total chlorophyll content can be used for the current state of forest stand and also input for various physiological vegetation models i.e. models for photosynthesis and evaporation etc.

The photosynthetic and carbohydrate status of *in vitro* cultured plants and *ex vitro* grown plants has been evaluated by many workers. In *Uniola* spp., Aracama et al. (2006) reported that starch utilization was co-related with development of leaves reporting more utilization with expanded leaf blades and generally *ex vitro* transfer exhibited significant decrease of starch and soluble sugars content in shoots. Another explanation for high levels of starch and TSS in the *in vitro* grown plants was the application of plant growth regulators in the plants growing in the *in vitro* conditions. These chemicals increased the TSS and the quality of shoots grown *in vitro* in the controlled environment as reported in *Syzygium* spp. (Moneruzzaman et al. 2011). Synthesis of biochemical components of organs ultimately leads to the formation of new structures and is fuelled by carbon and nitrogen assimilates (Lawlor, 2002). This also accounts for the utilization of amino acids like proline by the growing plantlets. Appropriate assimilation of biomolecules brings about the formation of new structures in plants (Niklas and Enquist, 2002) and thus accounting for more vigorous growth and low levels of proline content in the *ex vitro* grown plants. A common strategy of plants during *ex vitro* acclimatization is to use the carbohydrate storage of *in vitro*-produced leaves to cover metabolic demands of growing tissues (Fuentes et al. 2005). Proline plays multiple roles in the functioning of plants as depicted in the [Figure 2.1.7](#).

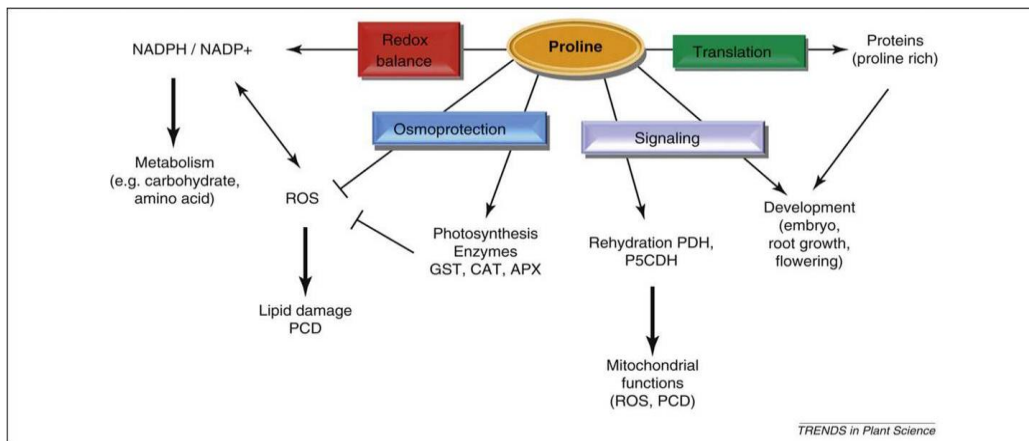


Figure 2.1.7 Multiple functions of proline in plants including metabolism, photosynthesis, developmental, PCD etc. Abbreviations: PCD: Programmed Cell Death; APX: Ascorbate Peroxidase; CAT: Catalase (*TRENDS in Plant Science*).

The anatomy of bamboo family has not been greatly investigated but earlier studies of Soderstrom and Ellis, (1988) and Brandis, (1907) have contributed considerably to anatomical data about several Bambusoideae species. The presence of epidermal cells containing silica bodies is an important character in Poaceae family (Metcalf, 1963). Observation of prickles has been mentioned by Renvoize, (1985) for Bambusoideae species. Mainly the studies were confined to leaf anatomy. Anatomical details of root and culm were not much available in literature. However, anatomical details of somatic embryos have been worked out by numerous workers. Histological analysis shows that non embryogenic cells are

large and vacuolated parenchymal cells whereas somatic embryos gradually develop from granular onsite to heart shaped, torpedo shaped and final cotyledons (Rao and Rao, 1988; Zhang et al. 2010 and Bag et al. 2012).

2.1.8 Molecular Characterization

Analysis of genetic fidelity of *in vitro* raised plants is of utmost importance in determining practicality of any micropropagation protocol. The phenotypic and genotypic variations may occur during *in vitro* culture and subsequently give rise to somaclonal variants (Kaeppler et al. 2000). Therefore, it is essential to assess the clonal fidelity of *in vitro* derived plants at regular intervals for the occurrence of any somaclonal variation (Chandrika et al. 2008 and Mehta et al. 2010). Among the various methods of *in vitro* propagation, the axillary shoot proliferation has been shown to be the safest method and least susceptible to genetic modification (Shenoy and Vasil, 1992; Agnihotri et al. 2009 and Negi and Saxena, 2010). However, the possibility of somaclonal variations cannot be ruled out even with this method. It is therefore extremely important to ascertain the clonal uniformity of the *in vitro* raised plants. Several techniques are available to assess tissue culture induced variations in plants such as morphological descriptions, physiological supervisions, cytological studies, isozymes and molecular markers (Gupta and Varshney, 1999 and Bhagat et al. 2012). However, the molecular markers are regarded as rapid, sensitive and more reliable alternative approach (Sharma et al. 2008 a, b). Several DNA markers viz. Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) have been employed to assess the genomic stability in regenerated plants (Mehta et al. 2010).

Though there are several reports on tissue culture of bamboos, there are only a few reports confirming the clonal fidelity of bamboo plantlets raised through axillary branching. Earlier, Das and Pal, (2005b) established the clonal fidelity of regenerants of *Bambusa tulda* and *B. balcooa* using only four RAPD markers. Inter Simple Sequence Repeats (ISSR) marker assay was employed to validate the clonal fidelity of *in vitro* raised *B. balcooa* plantlets (Negi and Saxena, 2010 and Brar et al. 2014). In *Dendrocalamus asper*, Singh et al. (2012 b) used 22 RAPD, 24 ISSR, 21 SSR and 11 AFLP markers for confirming the genetic uniformity among the plants established from nodal explants. Using AFLP technique, Mehta et al. (2010) demonstrated no major genetic variation in the *in vitro* regenerated shoots of *Bambusa nutans* showing 98.8% conservation and high level of genetic stability. Negi and Saxena, (2010) obtained 93 scorable bands with 15 ISSR markers and confirmed the genetic

uniformity of tissue culture raised progenies upto 27 passages in *B. nutans*. In *Guadua angustifolia*, Nadha et al. (2011) reported that all the banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant, thus ascertaining the true nature of the *in vitro* raised plants. In *Bambusa bambos*, Anand et al. (2013) reported no variation among the *in vitro* raised progeny and the mother plant in the banding profiles generated by total of fifteen RAPD and ISSR markers.

Table 2.1. Reports on Tissue culture of Bamboos species

Genus / Species	Explant type	Response	Reference
<i>Bambusa arundinacea</i> or <i>Bambusa bambos</i>	Seeds	Somatic Embryogenesis Callus: $N_6 + 3 \times 10^{-5}$ M 2,4-D Germination: BAP 5×10^{-5} M + 2,4-D 3×10^{-5} M + 250 mg/l PVP	Mehta et al. (1982)
	Nodal Explant	Multiple shoot formation	Nadgir et al. (1984)
	Young leaf	Callus: MS + 3 to 6 mg/l 2,4-D Plantlets: MS + 2 mg/l BAP + 0.5 mg/l NAA	Vasana, (1985)
	Inflorescence with Panicle and Spikelet	Precocious Flowering: White's medium + 0.5 ppm BAP + 5% CM	Nadgauda et al. (1990)
	Seeds	Multiple Shoot Formation: MS + 1 mg/l NAA + 4 mg/l BAP	Vasana, (1985)
	Seedlings	<i>In vitro</i> flowering: 2.2 μ M BAP + 5% CM + 2 % sucrose	Nadgauda et al. (1997 b)
	Seedlings	Germination: $\frac{1}{2}$ MS with no growth regulator <i>In vitro</i> flowering: 2.2 μ M BAP	Joshi and Nadgauda, (1997)
	Nodal Segments	Multiple shoot formation :3 mg/l BAP Rooting: 3 mg/l NAA for roots <i>In vitro</i> flowering: 3 mg/l BAP + 3 mg/l NAA	Arya and Sharma, (1998)
	Seeds	Multiple shoots: $B_5 + 10^{-5}$ M BAP <i>In vitro</i> flowering: Somatic Embryogenesis Compact callus: $B_5 + 2,4-D 2 \times 10^{-5}$ M + BAP 10^{-5} M Maturation: $GA_3 10^{-6}$ M + ABA 5×10^{-6} M + Ethephon 10^{-3} M.	Rao and Rao, (1988)
	Seeds	Germination: GA_3 (10 and 100 μ M) IAA (10 and 100 μ M)	Vamil et al. (2010)
	Young shoots and Nodal explants	Multiple Shoots: 2 μ M BAP + 5 μ M Kn Rooting: 1.5 μ M IAA and 1 μ M IBA	Nayak et al. (2010)
	Caryopses	Multiple Shoots: 5.0 μ M BAP + 2% Sucrose Rhizome: (2.5, 5.0 BAP + GA_3 0.1+ 50 NAA) μ M + 5 % sucrose	Kapoor and Rao, (2006)
	Nodal Explant	Multiple Shoots: 4.4 μ M BAP + 1.16 μ M Kn Rooting: 9.80 μ M IBA	Anand et al. (2013)
<i>Bambusa balcooa</i>	Nodal Explant	Multiple shoots: 11.25 μ M BAP + 4.5 μ M Kn Rooting: 1.0 μ M IBA	Das and Pal, (2005a)
	Nodal Explant	Bud Break: MS + 2.5 mg/l BAP + 1 mg/l Kn Multiple Shoots: on same medium Rooting: $\frac{1}{2}$ MS (Liq) + 0.2 mg/l IBA	Das and Pal, (2005b)
	Nodal Explant	Multiple Shoots: $\frac{1}{2}$ MS + 4×10^{-6} M BAP + 10^{-5} M Kn Rooting: 5×10^{-6} M NAA or 10^{-6} M Coumarin	Roohi et al. (1991)
	Mature Pseudospikelets	Somatic Embryogenesis: 1 mg/l 2,4-D + 0.16 % gelrite Regeneration: 5 mg/l BAP initially and 1 mg/l BAP later on	Gillis et al. (2007)
	Nodal Explant	Multiple shoots: 1.0 mg/l BAP Rooting: 1.0 mg/l BAP + 3.0 mg/l NAA	Mudoi and Borathakur,(2009)
	Nodal Explant	Multiple shoots: 1.5 mg/l BA Rooting: 3.5 mg/l NAA	Sharma and Sarma, (2011)
	Nodal Explant	Shoot Multiplication: MS media + BAP Rooting: $\frac{1}{2}$ MS + Auxins	Negi and Saxena, (2010)
	Nodal buds	Multiple Shoots: 1-5 mg/l BAP	Islam and Rahman,

		Rooting: 1-3 mg/l NAA + 1-5 mg/l IBA	(2005)
	Nodal explant	Multiple Shoots: 4.4 μ M BAP + 0.53 μ M NAA Rooting: Full strength MS + 16.11 μ M NAA	Brar et al. (2014)
<i>Bambusa beecheyana</i>	Inflorescence	Somatic Embryogenesis Callus: 3.0 mg/l 2,4-D + 2 mg/l Kn + 60 mg/l Sucrose Germination: same medium as for initiation or auxin free medium	Yeh and Chang, (1986 b)
	Roots	Embryogenic Calli: MS + CH 1 g/l + sucrose 30-60 g/l + Kn 2 mg/l + 2,4-D 3 mg/l	Chang and Lan, (1995)
<i>Bambusa edulis</i>	Nodal Explants	Multiple shoots: 0.1 mg/l TDZ Rooting: 0.1 mg/l TDZ + 0.5 mg/l 2,4-D <i>In vitro</i> Flowering on the same media	Lin and Chang, (1998)
	<i>In vitro</i> spikelets	Somatic Embryogenesis/ <i>In vitro</i> Flowering Germination: 0.5 μ M TDZ + 30 mg/l Sucrose Rooting and Flowering: 26.9 μ M NAA	Lin et al. (2003)
	Shoots, Nodal and Internodal segments	Somatic Embryogenesis Callus: 9.2 μ M Kn + 13.6 μ M 2,4-D Additives: 0.046 μ M TDZ + 6% sucrose + 0.1 % CM	Lin et al. (2004 a)
	Inflorescence	Inflorescence Proliferation: 0.01 to 0.1 mg/l 2.2 g/l gelrite was used	Lin et al. (2004 b)
<i>Bambusa oldhamii</i>	Inflorescence	Shoot proliferation and Flowering: 5 mg/l NAA + 1 mg/l ACC	Lin et al. (2005)
	Young Inflorescence	Somatic Embryogenesis Embryogenic callus: 3 mg/l 2,4-D+2 mg/l Kn + 60 mg/l Sucrose Germination: same medium as for initiation or auxin free medium	Yeh and Chang, (1986 a)
	Shoot tip	Plantlets: MS + 1 mg/l BAP	Huang et al. (1989)
	Nodal Segment	Multiple shoots: 0.45 μ M TDZ Rooting: 26.85 μ M NAA	Lin et al. (2007a)
	Nodal Explants	Multiple Shoot formation and Rooting	Banik, (1987)
	Seeds and Nodal explants	Multiple Shoots: MS (Liquid) + 4.4 μ M BAP Rooting: $\frac{1}{2}$ MS + 9.84 μ M IBA + 2.69 μ M NAA	Thiruvengadam et al. (2011)
<i>Bambusa glaucescens</i>	Nodal Segments	Multiple Shoots: 5 μ M BA + 15 μ M Kn Rooting: 25 μ M IBA	Shirin and Rana, (2007)
<i>Bambusa multiplex</i>	Shoot tip	Callus Induction: NAA + BAP Regeneration of plantlets on the same media	Huang et al. (1989)
<i>Bambusa tulda</i>	Nodal Explants	Multiple Shoots: MS (Liq)+12 BAP+0.1 IAA+100) μ M Glutamine	Mishra et al. (2008)
	Nodal Explants	Bud break: MS+2.0 mg/l BAP+1 mg/l Kn+8% coconut water Multiple Shoots: MS (Liq)+3 mg/l IBA Rooting: $\frac{1}{2}$ MS (Liq)+0.2 mg/l IBA	Das and Pal, (2005b)
<i>Bambusa nutans</i>	Seeds	Multiple Shoots: 0.5 mg/l BA Rooting: 0.5 mg/l IBA	Yasodha et al. (1997)
	Nodal explants	Somatic Embryogenesis: Callus Induction: 5.0 mg/l 2,4-D Germination: MS+1.0 mg/l (BAP + 2,4-D) +20 mg/l ascorbic acid	Mehta et al. (2010)
	Nodal explants	Shoot Proliferation: (13.2 BA+2.32 Kn+0.98 IBA) μ M Rooting: $\frac{1}{2}$ MS + (9.8 IBA+2.85 IAA+2.68 NAA) μ M	Negi and Saxena, (2011b)
	Nodal explants	Multiple Shoots and Rooting	Yadav et al. (2008)
	Culm cuttings	Multiple shoots: 2.22 μ M BA + 2% sucrose Rooting: 49.0 μ M IBA + 88 mM glucose	Yasodha et al. (2008)
	Nodal explants	Bud break: 7.5 μ M BAP Multiple Shoots: 5.0 μ M BAP Rooting: 10 μ M IBA	Sharma and Kalia, (2012)
<i>Bambusa vulgaris</i>	Nodal segments	Multiple Shoots: 0.2 BAP mg/l +0.05 IAA mg/l +5% CM Rooting: 0.5 BAP mg/l+ 0.2 Kn mg/l + 10% CM	Nadgir et al. (1984)
	Seeds	Somatic Embryogenesis and <i>In vitro</i> flowering Callus Induction: (3.0 2,4-D+0.25 Kn)mg/l Regeneration: $\frac{1}{2}$ MS+(0.5 Kn+10Ads+2.0 2,4-D)mg/l <i>In Vitro</i> Flowering: (0.5 Ads+0.25 IBA+0.5 GA ₃)mg/l	Rout and Das, (1994)
		Somatic Embryogenesis:	

	Nodal explants	Callus Induction: (2.2 BAP+9.04 2,4-D+14.76 IBA) μ M Shoots: 13.33 μ M BAP + 1.23 – 2.46 μ M IBA Rooting: $\frac{1}{2}$ MS + 0.49 IBA + 2% Saccharose	Rout and Das, (1997)
	Adult Field Culms	Multiple Shoots: 4 mg/l BA Rooting: 0.5 mg/l TDZ pretreatment and IBA 3mg/l	Ramanayake et al. (2006)
	Nodal Segments	Multiple Shoots: MMS + 2 mg/l BAP Rooting: MMS + 20 mg/l IBA	Ndiaye et al. (2006)
	Nodal Segments	Bud Break: Cytokinins Multiple Shoot Formation: GA ₃ Rooting: MS basal+50% macroelements + IBA 0.25 μ M	Hirimburegama and Gamage, (1995)
<i>Bambusa ventricosa</i>	Nodal Segments	Multiple Shoot formation: MS+3-4 mg/l BA+0.1-10 mg/l NAA 0.3% activated charcoal	Dekkers and Rao, (1989)
	Culm internodes and Culm sheath bases	Organogenic Callus: 0.5 to 5.0 mg/l 2,4-D Rhizogenesis: On same medium	Dekkers and Rao, (1989)
	Nodal Segments	Embryogenic Callus	Gielis, (1999)
	Shoot Apices	Shoots: 4.4 μ M BA Roots: 5.4 μ M NAA+0.44 μ M BA	Huang and Huang, (1995)
	Nodal Segments	Shoots: 1 mg/l 2,4-D + 1 mg/l Kn Somatic Embryogenesis Callus Induction: 1.5 mg/l BAP + 0.2 mg/l IBA Regeneration: WPM + 0.5 mg/l BAP	Cheah and Chaille, (2011)
<i>Bambusa glaucescens</i>	Axillary Buds and Leaves semi thin Transverse sections	Bud elongation and Rooting: 20 μ M NAA + 500 mg/l PVP Callus Induction: 18 μ M 2,4-D Embryos: 9 μ M 2,4-D+2.85 μ M IAA+2.2 μ M BA + 0.6 μ M ABA	Jullien and Van, (1994)
<i>Bambusa nana</i>	Axillary buds	Multiple shoots: 5.0 μ M BAP Rooting: MS (full strength) + 25 μ M IBA	Shirin and Arya , (2003)
<i>Bambusa wamin</i>	Nodal Segments	Multiple Shoots: 2.0 mg/l BAP + 0.8 mg/l Kn Rooting: 7.5 mg/l IBA	Arshad et al. (2005)
<i>Bambusa pallida</i>	Nodal Segments	Multiple Shoots: MS (Liq)+ 50 mg/l ascorbic acid +25 mg/l citric acid + 25 mg/l cysteine + 1.34 μ M NAA + 1.125 μ M TDZ Rooting: $\frac{1}{2}$ MS +2% sucrose + 1% glucose + pulse treatment of 0.5 mg/ml IBA for 30 min. prior to inoculation	Beena and Rathore, (2012)
<i>Dendrocalamus brandisii</i>	Zygotic Embryo	Multiple Shoots: MS + 6x10 ⁻⁵ M BAP Rooting: 0.5 - 2x10 ⁻⁵ M BAP + 1x10 ⁻⁵ M NAA	Vongvijitra, (1988)
	Zygotic Embryo	Flowering: MS + 0.5 ppm BAP + 5% CW + 0.5 ppm IBA	Nadgauda et al. (1990)
	Nodal Segments	Multiple Shoot formation: 5 mg/l BAP Rooting: 1.0 mg/l IBA after treatment in 100 mg/l IBA sol ⁿ 24 hrs	Mukunthakumar et al. (1999)
<i>Dendrocalamus asper</i>	Nodal Segments and Seeds	Multiple Shoot formation: 3 mg/l BA Rooting: 3.0 mg/l NAA	Arya et al. (2002)
	Inflorescence	Multiple Shoot formation and Rooting	Arya et al. (2008a)
	Nodal and Leaf Bases	Callus induction: 30 μ M 2,4-D+2% sucrose Proliferation: 9 μ M 2,4-D+2.85 μ M IAA+0.88 μ M BAP Maturation: 6% sucrose or 5 μ M ABA Plantlets: 4.4 μ M BAP+2.8 μ M GA ₃ with 70% conversion rate	Arya et al. (2008b)
	Nodal Explants	Organogenesis: Callus: 1 mg/l (2,4-D+BAP) Multiple Shoots: 7.0 mg/l BAP Rooting: 1 mg/l (2,4-D+IAA+NAA)	Ali et al. (2009)
	Nodal Explants	Shoot Multiplication: MS+(10 BAP+75 Ads) μ M Rooting: $\frac{1}{2}$ MS+(IBA+NAA) μ M	Singh et al. (2012b)
	Nodal Explants	Shoot Multiplication: MS + 5 mg/l BAP + 40 mg/l Ads Rooting; MS + 1 mg/l IBA	Banerjee et al. (2011)
	Internodal segments	Multiple shoots and Rooting: 2.0 mg/l BA Callus: (0.5Kn+3.0 NAA) mg/l	Shroti et al. (2012)
	Nodal explants	Multiple shoot formation: 8.8 μ M BAP + 13.5 μ M adenine sulfate Rooting: 14.76 μ M IBA + 3.67 μ M NAA	Nadha et al. (2013)
		Multiple Shoots :2 mg/l BAP + 0.1 mg/l Kn + 1 g/l Benlate	Ramanayake and

<i>Dendrocalamus giganteus</i>	Nodal Segment	Rooting: 3 mg/l IBA + 10 mg/l Coumarin	Yakandawala, (1997)
	Axillary Shoots, Spikelets and Roots	Organogenesis Callus: 33.9 μ M 2,4-D + 16.1 μ M NAA + 4% sucrose	Ramanayake and Wanniarachchi, (2003)
	Mature Seeds	Somatic Embryogenesis: 13.6 μ M 2,4-D	Rout and Das, (1994)
	Nodal Explant	<i>In vitro</i> Flowering: 26.6 μ M BAP + 2 % sucrose	Ramanayake et al. (2001)
	Nodal Explant	Multiple Shoots: 20 μ M BAP Rooting: 25 μ M IBA+0.05 μ M BAP	Arya et al. (2006)
	Nodal Segments	Multiple Shoots: ½ MS + 26.6 μ M BAP + 0.46 μ M Kn + 9 % CM Rooting: Failed Callus induction: 4.4 μ M BAP + 4.52 μ M 2,4-D + 5.37 μ M NAA Caulogenesis: 11.10 μ M BA + 2.32 μ M Kn Rhizogenesis: 98.41 μ M IBA	Yasodha et al. (2010)
	Seeds	Shoot multiplication: GA ₃ + BAP Callus Induction/Proliferation: 3 mg/l 2,4-D + 0.5 mg/l Kn Shoots Regeneration: 1 mg/l NAA + 0.5 mg/l Kn Rooting: 5 mg/l IBA	Devi et al. (2012)
	Nodal Segments	Bud Break: 7 mg/l BAP	Rajapakse et al.(1991)
<i>Dendrocalamus hamiltonii</i>	Nodal Segments	Shoot bud differentiation: (BAP + 2,4 -D 1 mg/l each) Rooting (IBA or NAA 0.5 mg/l each) Callus (2,4-D & BAP 1 mg/l each) Shoot buds differentiation (GA ₃ 0.5 mg/l) Rhizome :(IBA/NAA 0.5 mg/l) and Coumarin/ Choline Chloride	Sood et al. (1992)
	Nodal Segments and Seeds	Shoot proliferation: nodal segments: (1.0 mg/l BAP) Rooting: nodal segments: 20 mg/l IBA pulse Shoot Proliferation: Seeds: 7.0 mg/l BAP Rooting: Seeds: 20 mg/l IBA pulse	Arya et al. (2009)
	Seedlings	Multiple shoots: 4.4 μ M BA Rooting: occurred in Multiple shoot media only <i>In vitro</i> flowering: 2.2 μ M BA + 5% CM	Chambers et al. (1991)
	Nodal Explants	Multiple Shoots: ½ MS + 2.5 mg/l BA Rooting: ½ MS + NAA 0.5 mg/l+0.9 mg/l Choline Chloride + IBA 1 mg/l + Coumarin 9 mg/l Embryogenic Callus: BA + 2,4-D (1 mg/l each) + GA ₃	Sood et al. (2002a)
	Nodal Explants	Axillary Bud Break: ½ MS + 3% sucrose and no PGR Shoot multiplication and Rooting Somatic Embryogenesis using 2,4-D	Sood et al. (2002b)
	Nodal Explants	Callus Induction: ½ MS+ 1 mg/l (2,4-D+BAP+NAA) Maturation of Embryos: ½ MS + 2.5 mg/l BAP	Godbole et al. (2002)
	Nodal Explants	Multiple Shoots: 8.0 μ M BAP + 1.0 N μ M NAA Rooting: 100 μ M IBA	Agnihotri and Nandi, (2009)
	Nodal Explants	Multiple Shoots: MS + 8 μ M BAP + 1 μ M NAA Rooting: 100 μ M IBA	Agnihotri et al. (2009)
	Zygotic Embryo	Somatic Embryogenesis and Organogenesis Callus Induction: 1-3 mg/l 2,4-D Shoots: 2 mg/l BA + 1 mg/l Kn + 1 mg/l NAA Rooting: 5 mg/l IBA	Zhang et al. (2010)
	Nodal Explants	Somatic Embryogenesis Induction and Shoots: BAP 5.0 μ M + 2,4-D 7.5 μ M Rooting: ½ MS + IBA 5.0 μ M	Bag et al. (2012)
	Seeds	Multiple Shoots: 10 μ M BAP Rooting: 25-150 μ M IBA	Arya et al. (2012)
	Seeds/ Nodal explants	Multiple shoots: Whites medium + BA (2 mg/l) <i>Ex vitro</i> and <i>in vitro</i> rooting using IBA and NAA	Jha et al. (2013)
	Internode	Callus Initiation: 1 ppm 2,4-D (Compact) Shoots: 1 ppm 2,4-D + 1 ppm BAP	Zamora et al. (1988)

<i>Dendrocalamus latiflorus</i>	Seedlings	Multiple Shoot Formation and Rooting	Yuxia and Guangchu, (2000)
	Inflorescence	Embryogenic calli: (3.0 2,4-D+2 Kn+250 PVP) mg/l+ 1% CM Multiple Shoots: 0.1 mg/l TDZ Rooting: NAA 5 mg/l <i>In vitro</i> flowering: TDZ 1 mg/l	Lin et al. (2007 b)
<i>Dendrocalamus longispatus</i>	Nodal Explant	Multiple Shoots: BAP (1.5×10^{-5}) + IBA (1×10^{-6}) M+10% CM Rooting: IAA (1.0×10^{-5}) + IBA (1×10^{-5}) M	Saxena and Bhojwani, (1993)
	Nodal Explants	Somatic embryogenesis & direct organogenesis Callus: MS/B ₅ (2,4-D & 2,4,5-T at 1 to 10 mg/l) Embryoids: 1/2 MS + 5 mg/l 2,4,5-T (roots)	Saxena and Bhojwani, (1991)
<i>Dendrocalamus membranaceus</i>	Seeds	Multiple Shoots: 0.5 mg/l BA Rooting: 0.5 mg/l IBA	Yasodha et al. (1997)
	Seeds	Multiple Shoot Formation: 8.8 μ M BAP + 2.3 μ M Kn Rooting: On same medium as shoot proliferation	Brar et al. (2013)
<i>Dendrocalamus farinosus</i>	Seeds and Young Shoots	Somatic Embryogenesis Callus Induction: MS + (2.0 2,4,5-T + 0.2 Kn+ 0.4 IBA) mg/l Shoots: 2.5 mg/l Kn + 0.5 mg/l IAA Rooting: 0.4 mg/l IBA + 0.25 mg/l IAA	Hu et al. (2011)
<i>Dendrocalamus strictus</i>	Embryo	Embryo culture: White's medium	Alexander and Rao, (1968)
	Pollen Grains	<i>In vitro</i> Flowering White medium + 0.5 ppm BAP + 5 % CM	Nadgauda et al. (1990); (1993)
	Seedling	Multiple Shoot Formation and Rooting on MS medium with cytokinins and auxins.	Kumar, (1994)
	Seedling and Nodal Explants	Multiple Shoot Formation and Rooting	Nadgir et al. (1984)
	Seeds (Caryopses)	Somatic Embryogenesis: B ₅ Medium Germination: $\frac{1}{2}$ B ₅ + 5×10^{-7} M IBA + 10^{-7} M NAA + 1% sucrose	Rao et al. (1985)
	Seedlings	Multiple Shoot Formation and Rooting	Dekkers and Rao, (1989)
	Nodal explants	Multiple Shoot Formation: 0.5 mg/l IAA+15 mg/l Ads	Paranjothy et al. (1990)
	Excised embryos and seeds	Somatic Embryogenesis Callus induction: B ₅ medium + 10 μ M 2,4-D Proliferation of embryogenic callus: same medium or MS medium devoid of auxins and 85% formed plantlets when $\frac{1}{2}$ MS (micronutrients), coconut water and myoinositol is used	Zamora and Gruezo, (1990)
	Seedlings	Plantlets	Preetha et al. (1992)
	Seeds	Embryoids: 2,4-D 3 mg/l + 0.5 mg/l Kn Plantlets: NAA 1mg/l + 0.5 mg/l Kn Artificial seed encapsulation	Mukunthakumar and Mathur, (1992)
	Nodal Segments	Shoots: 0.5 mg/l IAA+ 15 mg/l Ads Rooting: 1 mg/l IBA+0.5 mg/l 2,4-D+1 mg/l phloroglucinol	Chaturvedi et al. (1993)
	Seeds	Callus Induction: (3.0 2,4-D+0.25 Kn)mg/l Regeneration: $\frac{1}{2}$ MS+(0.5 Kn+10Ads+2.0 2,4-D)mg/l <i>In Vitro</i> Flowering: (0.5 Ads+0.25 IBA+0.5GA ₃)mg/l	Rout and Das, (1994)
	Seedlings	Culture Initiation: $\frac{1}{2}$ MS + 0.5 mg/l BA + 2% sucrose Proliferation: MS + No PGR's	Shirgurkar et al. (1996)
	Seeds/ Seedlings	Multiple Shoot Formation	Maity and Ghosh, (1997)
	Nodal segments and Seedlings	Multiple Shoots: 0.25-2mg/l BA+0.5-1.0 mg/l Kn+200 ml/l CM Rooting: MS (liq)+ IBA Seedling on White's media with 2% sucrose	Ravikumar et al. (1998)
Seeds	Somatic Embryogenesis; Regeneration shoots: 1×10^{-5} M 2,4-D+		

		5x10 ⁻⁶ M Kn + 2x10 ⁻⁶ M IBA+ 250 mg/l PVP Rooting: ½ MS+3x10 ⁻⁶ M NAA+2.5x10 ⁻⁶ M IBA	Saxena et al. (1999)
	Shoots	Shoot proliferation: MS Liquid + 0.01 to 1.0 mg/l TDZ <i>In vitro</i> flowering: 0.5 mg/l TDZ No seed set and Incomplete Anthesis	Singh et al. (2000)
	Seedlings	Multiple shoot formation and Rooting	Singh et al. (2001)
	Seeds	Multiple Shoots: ½ MS + 2 mg/l BAP Rooting: ½ MS + 2 mg/l IBA	Reddy, (2006)
	Seeds Mature Clump	Seeds: Embryogenesis: 0.2 mg/l BAP + 5% Coconut Milk + 2% Sucrose Rooting: ½ MS + 2% Sucrose Embryogenesis: Thiamine HCl (40) + MS (major) Inositol (1000) + 2,4-D 3.5 % Mature Clump: 0.5 % Kn + BAP 1.0 % + CM (5%) Shoot Proliferation: (1.0 BAP mg/l +0.5 Kn mg/l) +5 % CM + 2 % sucrose + 200 mg/l CH	Mascarenhas et al. (1988)
	Inflorescence	Shoot Multiplication: 0.5 mg/l triacontanol + 3.0 mg/l BA Rooting: ½ MS + alcoholic rice bran 2.5 mg/l + 3 mg/l NAA	Mishra et al. (2001)
	Nodal segment	Multiple Shoots: 4 mg/l BAP+15 mg/l Ads Rooting: MS (full strength)+1 mg/l IAA+3 mg/l IBA +5 mg/l NAA	Pandey and Singh, (2012)
<i>Gigantochola atroviolaceae</i> Widjaja	Nodal Segment	Multiple Shoots: BAP 20 µM + NAA 3.0 µM Rooting: MS (full strength) + IBA 35.0 µM	Bisht et al. (2010)
<i>Guadua angustifolia</i>	Nodal Segment	Multiple Shoot Formation and Rooting (On same media containing BAP 5 mg/l)	Jimenez et al. (2006)
	Nodal explants	Multiple Shoot Formation and Rooting	Rathore et al. (2009)
	Nodal explants	Multiple Shoot Formation and Rooting	Marulanda et al. (2002)
	Nodal explants	Bud break: 2 mg/l BAP Multiple shoot proliferation: 2 mg/l BAP and 10 mg/l Ads Rooting: on same multiplication medium	Nadha et al. (2011)
<i>Melocanna baccifera</i>	Nodal explants	Multiple Shoots: MS (Liq) + 15 µM BAP + 3 µM Kn Rooting: ½ MS 25 µM IBA	Kant et al. (2009)
<i>Otatea acuminata aztecorum</i>	Seeds	Somatic Embryogenesis Callus: MS & B ₅ (3 mg/l 2,4-D + 0.5 mg/l BAP) Germination: B ₅ media with no Supplements	Woods et al. (1992)
<i>Oxytenanthera abyssinica</i> A. Rich. Munro	Nodal Explants	Multiple Shoots: MS + 5.0 mg/l BA + 0.2 mg/l NAA Rooting: MS + 8.0 mg/l IBA	Diab and Mohamed, (2008)
<i>Ochlandra wightii</i>	Seeds	Shoots: ½ MS + 0.5 mg/l BAP + 0.5 mg/l Kn Rhizome: Kn 1mg/l + 4% sucrose	Bejoy et al. (2012)
	Nodal Explants	Multiple Shoots: BAP 2.0 mg/l + 0.5 mg/l Kn Rooting: ½ MS + 0.5 mg/l IBA	
<i>Arundinaria callosa</i>	Nodal Explants	Multiple Shoots: 13.3 µM BAP + 1.0 µM IBA Rooting: ½ MS + 25µM IBA + 0.05µM BAP	Devi and Sharma, (2009)
<i>Arundinaria gigantea</i>	Nodal Explants	Multiple Shoots: 0.1 µM IBA + 0.01 µM TDZ Rooting: (-)	Baldwin et al. (2009)
<i>Phyllostachys aurea</i>	Shoot tip	Callus: 1-3 mg/l 2,4-D	Huang and Murashige, (1983)
<i>Phyllostachys meyeri</i>	Seedlings	Multiple Shoot Formation and Rooting on ½ MS liquid hormone Free media	Ogita et al. (2008)
<i>Phyllostachys nigra</i>	Shoots	Callus: ½ MS + 3 µM 2,4-D	Ogita, (2005)
<i>Phyllostachys pubescens</i>	Zygotic seed	Callus initiation: 4 mg/l 2,4-D + 1 mg/l Zeatin Regeneration: 5-7 mg/l Zeatin	

	embryos	Rooting: 2 mg/l NAA	Yuan et al. (2013)
<i>Phyllostachys bambusoides</i>	Nodal Explants Leaf Sheaths	Callus Induction: Picloram 8.0 mg/l Rooting: 2% glucose	Komatsu et al. (2011)
<i>Pseudoxytenanthera stocksii</i>	Nodal Explants	Multiple Shoots: MS + 2.68 μ M NAA + 4.4 μ M BAP Rooting: $\frac{1}{2}$ MS + 4.90 μ M IBA + 0.44 BA	Sanjaya et al. (2005)
<i>Sasa pygmaea</i>	Shoot Tip	Callus	Huang and Murashige, (1983)
<i>Schizostachyum brachycladum</i>	Culm Sheath	Organogenic Callus: 0.5 to 5.0 mg/l 2,4-D Rhizogenesis: On same medium	Dekkers and Rao, (1989)
<i>Sinocalamus latiflorus</i>	Seeds	Somatic Embryogenesis Callus: 6 mg/l 2,4-D Germination: 3 mg/l 2,4-D + 2 mg/l Kn & no PVP	Yeh and Chang, (1987)
	Anthers	Somatic Embryogenesis N ₆ 1 mg/l 2,4-D+1 mg/l BA+2 g/l charcoal + 9% sucrose	Tsay et al. (1990)
<i>Thyrostachys oliveri</i>	Nodal buds	Multiple Shoots: 1-5 mg/l BAP Rooting: 1-3 mg/l NAA + 1-5 mg/l IBA	Islam and Rahman, (2005)
<i>Thyrostachys siamensis</i>	Culm Sheath Base	Organogenic Callus: 0.5 to 5.0 mg/l 2,4-D Rhizogenesis: On same medium	Dekkers and Rao, (1989)
	Seeds	MS +1 mg/l NAA + 2 mg/L 6-BAP MS +2 mg/l NAA + 4 mg/L 6-BAP	Vasana, (1985)

3 Materials and Methods

3.1 Experimental Material

Three different edible bamboo species namely *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos* were selected for the present study.

Plant Profile

3.1.1 *Dendrocalamus membranaceus*



Figure 3.1.1 a. Culms of *D. membranaceus* **Figure 3.1.1.b.** Leaves **Figure 3.1.1 c.** Emerging young shoots **Figure 3.1.1 d.** A flowering shoot **e.** Seeds

Dendrocalamus membranaceus commonly known as White Bamboo or Waya bamboo is an edible tropical bamboo growing at an elevation of 400 m with $< 20^\circ$ slopes along the mountain sides and the streams. **Culm:** Culm usually grows straight attaining a height of 14-18 m with a diameter of 10-12 cm (Figure 3.1.1 a). Culm walls are 1-2 cm thick. **Culm Sheath:** Culm sheath is large, smooth and shining within, with dark brown hairs at the back. Auricles are wavy, smooth, stiff and end in a sharp point giving slightly blackish appearance. **Leaves:** Leaves are typically 10-12 cm long and 1-2 cm wide. Leaf tapers from a round bottom to the apex (Figure 3.1.1 b). **Shoots:** Young emerging shoots are light green in colour, possess high nutritional value and are edible after processing. They are visible emerging from the ground during the rainy season (Figure 3.1.1 c). Young shoots can be consumed fresh, fermented or canned. The juvenile shoots are not

only delicious but are rich in nutrient components mainly proteins, carbohydrates, minerals and fiber and low in fat and sugars. They also serve as good source of food for young elephants. **Inflorescence:** Pseudospikelet, 2-keeled inflorescence usually 2.5 to 5 cm in diameter (Figure 3.1.1 d). Flowering is sporadic and the flowering cycle is from 70 – 90 years. **Seeds:** Seeds are brown in colour, grain like covered with glumes having tapering ends (Figure 3.1.1 e). They lose their viability very soon and hence are stored at 4°C temperature.

3.1.2 *Bambusa balcooa*



Figure 3.1.2 a Culm of *B. balcooa* Figure 3.1.2 b. Leaves Figure 3.1.2 c. A young emerging culm. Figure 3.1.2 d. Flowering Branches.

It is commonly known as Balku bans or Borak bans. It prefers to grow in tropical monsoon climate with an average rainfall of 2500 to 3000 mm. **Culm:** Culm is erect, 17.5 to 30 m tall, 2.5 to 10 cm in diameter having dirty silvery brown appearance. Internodes are 20 to 45 cm long (Figure 3.1.2 a). **Culm sheath:** The culm sheath is deciduous, with a smooth inner surface, a brown hairy outer surface and a ciliate margin. The blade is triangular. Auricles are absent. **Leaf:** Leaf blade is oblong lanceolate, glabrous with ciliate margin (Figure 3.1.2 b). **Shoots:** Young shoot is blackish green and covered with blackish hairs. The new emerging shoots (Figure 3.1.2 c) of this clumping bamboo emerge very near the base of existing plant as opposed to running type of bamboos. This habit makes it a very dense bamboo. The shoots of this bamboo are not only delicious but are also rich in nutrients and low in fats and are consumed in fresh, fermented or roasted form. **Inflorescence:** Inflorescence is compound spikelet upto 1 m long (Figure 3.1.2 d). Flowering is isolated/rare and the flowering cycle is

from 55-60 years. The flowers are green with purple tips .**Seeds:** The clump/plant dies after flowering without setting any seed.

3.1.3. *Bambusa bambos*



Figure 3.1.3 a. Clumps of *Bambusa bambos* **Figure 3.1.3 b.** Leaves **Figure 3.1.3 c.** Emergence of a young shoot from ground **Figure 3.1.3 d.** A flowering Shoot **Figure 3.1.3 e** Seeds.

Bambusa bambos commonly known as “Giant Thorny” bamboo is a clumping bamboo of Southeast Asian countries. **Culms:** It has fast growing strong woody culm which is 20-30 m tall with an average diameter of 10-18 cm. Nodes are slightly swollen and internodes are dark green in colour (Figure 3.1.3 a). **Culm Sheath:** Culm-sheath is coriaceous, glabrous to pubescent with dark brown velvety hairs. **Leaves:** Leaf blade is linear, lanceolate, narrowed to an acuminate tip with mid-vein inconspicuous on the abaxial side and prominent on adaxial side. Leaves are 15-30 cm long and 8-15 mm broad (Figure 3.1.3 b). **Shoot:** Young shoots are bluish green or brown in colour. Shoots can be considered as vegetables and prepared to make food (Duriyaprapan and Jansen, 1995). The shoot shells, a by-product of the industrial processing of canned bamboo shoots are available as both fresh and boiled material and can be used as feedstuffs (Liu et al. 2000) (Figure 3.1.3 c). **Inflorescence:** It is a

pseudospikelet, bractiferous, clustered at nodes in untidy tufts. This bamboo species flowers gregariously after every 40-60 years (Figure 3.1.3 d). **Seeds:** Seeds are light brown in colour with pointed ends about 0.5 – 0.6 cm long (Figure 3.1.3 e). They appear like wheat grains covered with glumes. *Bambusa bambos* seeds are generally viable for a period of 6-8 months. A single clump gives 60-100 kg of seeds and about 70,000 to 85,000 seeds weigh one kilogram.

3.2 Experimental Requirements

3.2.1 Chemicals and Reagents

The chemicals used for tissue culture work were from Hi-Media/Sigma/Sd fine brands (AR grade). All the reagents used for biochemical analyses were of extra pure analytical grade. For molecular characterization, *Taq* buffer, *Taq* polymerase, dNTPs were procured from Bangalore Genei Pvt. Ltd, Bangalore, India. RAPD and ISSR primers were procured from Sigma-Aldrich, Bangalore, India.

3.2.2 Plasticware/Glassware

Plasticware (Tarsons Products Pvt. Ltd, Kolkata) included autoclavable narrow and wide mouth bottles (500 ml), wash bottle (500 ml), graduated measuring cylinders (100, 500 and 1000 ml), utility trays, test tube baskets, tips (10, 200, 1000 µl), micro tip box (0.2-10 µl, 2-200 µl, 200-1000 µl), syringe filter (25 mm), micro centrifuge tubes (0.5, 1.5, 2 ml), oak ridge centrifuge tubes (10, 30, 50, 70ml), float rack (16 places), centrifuge tube box (36 places), test tube stand, test tube cap (25 mm), acupipet (0.5-2, 10-100, 100-1000 µl), PCR tubes (Tarsons) and biohazardous waste container (5 lts). All glassware was made up of borosilicate glass (Borosil glass Ltd. Mumbai). The glassware used for experimental work included conical flasks (100 ml, 150 ml, 250 ml, 500 ml, 1000 ml and 2000 ml), test tubes (25 x 125; 25 x 150 mm), beakers (100, 200, 250, 500 and 1000 ml), glass pipettes (1 ml, 2 ml, 5 ml, and 10 ml), round bottle flask (500 ml and 1000 ml), amber wide mouth bottles (500 ml), reagent bottles (100, 200 and 500 ml), dessicator vacuum, graduated cylinders, volumetric flask, stirring rod (16 x 16 mm) and Petridishes. Tissue culture Jam bottles (Kasablanca, Mumbai) were also used for routine subculture work. Laboratory consumables included tissue roll, cotton, aluminum foil, surgical blades, disinfectants, forceps etc.

Before use, the glassware were thoroughly brushed with alkaline detergent teepol (10 %) and then washed in running tap water. These were then treated with chromic acid for 24 hours, followed by thorough washing under running tap water. Distilled water was poured into every culture vessel, which was tightly plugged. Plugs were made out of non-absorbent

surgical cotton wrapped in muslin cloth. Glassware were steam sterilized in an autoclave at a pressure of 1.1 kg/cm² for 15 - 20 minutes

3.2.3 Instruments

pH meter and Conductivity meter (Cyberscan, China), weighing balance (Afcoset), Microwave oven (LG), BOD Incubators (NSW), Orbital shakers (Orbitek), Horizontal shakers (Labcon), Air conditioners (Voltas), Heater (Lexus, USHA), Vortex (SPINIX), Refrigerator (LG), Deep Freezer (Vestfrost), Autoclave (Equitron), Laminar Flow Chambers (Klenzaid), Microtome (Shandon Finesse), Water bath incubator NSW-133 (Narang Scientific Works), Centrifuge (Plastocrafts), Magnetic stirrer (Tarsons), Hot air oven (Narang Scientific Works), Sonicator (Sonics), UV-Vis Spectrometer (Biotek Synergy, HT) and Gel Doc System (Molecular Imager, Gel DocTM XR Imaging System), Light Microscope (Lobophot, Nikon, Japan), Scanning Electron Microscope (S 34 00 N, Hitachi, Japan) were used during the course of present experimentation.

3.3 Establishment of Micropropagation Protocol

3.3.1 Explant Source and Selection of Explants

Explants were collected from the elite field grown plants of all the three species viz. *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos* growing in the growing in experimental farm, CSIR-Institute of Himalayan Bioresource Technology, Palampur, India at an elevation of 1300 masl. and 32°7'11' N latitude and 76°31'48'' E longitude. Elite mother plants were selected on the basis of four important characteristics 1) height of bamboo culms 2) girth of culms at third internode from the bottom 3) number of culms per clump and 4) length of internode. Seeds of *D. membranaceus* were procured from China and used in the present study.

Single node segments (3-4 cm long) were collected from healthy field growing culms of *Dendrocalamus membranaceus* (1 year old), *Bambusa balcooa* (4 year old) and *Bambusa bambos* (5 year old) Seeds for the present experimentation were procured from China which were more than 6 months old i.e. ageing seeds. Besides nodal segments and seeds, the apical buds and leaf explants were also tested for initiating cultures which however failed to respond under *in vitro* conditions.

3.3.2 Surface Sterilization of Explants: After removing the leaf sheaths carefully, the nodal segments and seeds were rinsed with 0.01% Tween 20 (Polyxyethylene sorbitan monolaurate, Qualigens, Mumbai) for 10 minutes. Explants were given treatment with bavistin (0.25% w/v) first, followed by streptomycin sulphate (0.25%) and tetracycline (0.01 to 0.1%) for 15 to 30 minutes. The explants were further washed with 70 % alcohol for 1 minute followed by

treatment with sodium hypochlorite (15%) for 10 minutes for seeds and 8 minutes for nodal explants respectively. Final disinfection was done with an aqueous solution of 0.1% mercuric chloride (HgCl_2) for 8 minutes for nodal explants and 10 minutes for seeds in a laminar air flow cabinet. Each treatment was followed by repeated washings with sterile distilled water. Explants were then inoculated on Murashige and Skoog's (1962) medium containing 1 to 3% sucrose, 0.8% agar and various growth regulators. Portions of nodal explant exposed to sterilant were removed with the help of a sharp and sterile secateur.

3.3.3 Culture incubation: Cultures were incubated under cool, white 40 W fluorescent lamps with irradiation at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) at a temperature of $25 \pm 2 \text{ }^\circ\text{C}$ with 16/8 hr light : dark cycle. Seeds were kept in dark chambers initially under same *in vitro* conditions. Seeds were also maintained under *ex vitro* in BOD incubators at temperature range of 5, 10, 15, 25, 30 and $35 \text{ }^\circ\text{C}$ respectively.

3.3.4 Nodal Explant Culture

3.3.4.1 Effect of Season and Source of Explant: Nodal explants were collected from January to December, 2009 on a regular basis to study the effects of seasons on the desired response of the explant. Twenty four explants were inoculated after every 15 days. The explants were collected from lower five nodes of the mature culms. Nodal explants were inoculated onto MS basal medium or MS medium supplemented with different cytokinins (as per requirement), 1% - 3% sucrose (as per requirement) and 0.8% agar for initial screening. Percent survival was tested on tenth day basically for checking asepsis and bud break.

3.3.4.2 Shoot Multiplication

For shoot multiplication, the sprouts were excised and transferred to MS medium containing different plant growth regulators. Combinations and permutations of auxins (low concentration) and cytokinins were tested for their efficiency for shoot multiplication. The *in vitro* raised shoots were divided into clumps of 3, 6 and 9 and their effect on multiplication was studied by sub-culturing to fresh medium at a regular interval of 4 weeks. Details of the PGR combinations used for shoot multiplication are given below:

1. MS + BAP (1.1 to $17.6 \mu\text{M}$)
2. MS + BAP (1.1 to $17.6 \mu\text{M}$) and Kn (1.16 to $9.3 \mu\text{M}$)
3. MS + BAP (1.1 to $17.6 \mu\text{M}$) + NAA (1.34 to $2.2 \mu\text{M}$)
4. MS + Kn (1.16 to $9.3 \mu\text{M}$)
5. MS + Kn (1.16 to $9.3 \mu\text{M}$) + NAA (1.34 to $2.2 \mu\text{M}$)

3.3.5 Rooting of Microshoots

Shoots were excised in clumps of 3-4 from multiple shoot bunches and transferred onto MS media in full, half and quarter strengths supplemented with NAA (2.68 to 24.16 μM), IBA (2.45 to 24.5 μM) and BAP (1.1 to 4.4 μM) either alone or in combination with each other. Either 0.8% agar or 0.2% gelrite was used for induction of roots in all the three species. PGR combinations used for root induction are given below:

1. MS + NAA (2.68 to 24.16 μM)
2. MS + IBA (2.45 to 24.5 μM)
3. MS + NAA (2.68 to 24.16 μM) + BAP (1.1 to 4.4 μM)
4. MS + NAA (2.68 to 24.16 μM) + 9 mg/l Coumarin
5. MS + IAA (1.42 to 2.85 μM)

3.3.6 Acclimatization and *Ex Vitro* Transfer

The rooted plantlets were removed from the culture tubes and washed in lukewarm water to remove the agar traces sticking to them. Plants were transferred to plastic pots (7''x 5'') containing wet sand and were placed in the growth room for 2 weeks. They were covered with perforated plastic covers/jars to protect the loss of water. Thereafter, the plantlets were transferred to polybags containing suitable potting mixture and were shifted to greenhouse for acclimatization. Various potting mixture used were given below:

PM₁ - sand: soil: farmyard manure:: 1:1:1

PM₂ - sand: soil: farmyard manure:: 1:1:2

PM₃ - sand: soil: farmyard manure:: 1:0:0

PM₄ - sand: soil: farmyard manure:: 0:0:1

Tissue culture raised plants of all the three species were first kept in the green house for hardening and retained there for long (5-6 months) because of adverse environmental conditions of winter months outside. The plants were regularly monitored and sprayed with Hogland solution every 7 days. The best time for their transfer to fields was the beginning of the rainy season i.e. June end or July when they exhibited high survival percentage. Hardened plants were transplanted in the field to pits (2ft×2ft×2ft) at a plant to plant and row to row distance of 6 meters.

3.3.7 Callus Induction and Proliferation

The *in vitro* raised shoots were excised into small pieces (0.5-1cm) and transferred onto MS medium supplemented with various PGR combinations and the medium without PGRs served as a control. The medium was gelled with 0.8% agar and supplied with 3% sucrose unless

otherwise mentioned and the details of combinations employed for callus induction and proliferation are as follow:

1. MS + 2,4-D (1.13-18.12 μ M)
2. MS + NAA (1.34-24.16 μ M)
3. MS + BAP (1.1-17.6 μ M)
4. MS + 2,4-D (1.13-18.12 μ M) + NAA (1.34-24.16 μ M)
5. MS + 2,4-D (1.13-18.12 μ M) + BAP (1.1-17.6 μ M)

3.3.8 Differentiation from the Callus

Callus was shifted to various combinations of cytokinins and auxins for inducing differentiation of shoots and roots and best combinations for optimum response were selected.

3.3.9 Seed Culture

3.3.9.1 Germination

The seeds used for micropropagation studies were procured from China and were more than 6 months old i.e. ageing seeds. Seeds were surface sterilized and inoculated onto MS medium for germination studies.

3.3.9.2 Viability Test for Seeds

Seeds were sectioned longitudinally and divided in three lots and subjected to 2, 3, 5 Triphenyl Tetrazolium Chloride (TTC) test. Seeds were dipped in an aqueous solution 0.1% of TTC for 24 hours at 30°C. They were then kept in dark as outlined by International seed testing association, ISTA (1985).

Percentage viability (V) was taken as:

$$V = \text{No of viable seeds} / \text{Total no seeds tested} \times 100.$$

3.3.9.3 Effect of Light

Seeds were incubated under continuous dark and under light conditions with a photoperiod of 16 h light/8 hr dark for studying the effect of light on germination. Emergence of radicle was taken as a sign for initiation of germination.

3.3.9.4 Effect of Temperature

The sterilized seeds were incubated at different temperatures of 5, 10, 25, 30 and 35 ° C for recording the optimum temperature for germination. Results were recorded after regular time intervals of 20 days.

3.3.9.5 Effect of Gibberellic Acid (GA₃)

Effect of gibberellic acid (GA₃) concentrations viz. 10 to 70 ppm for enhancing seed germination was studied. Seeds were soaked in solution of GA₃ and kept overnight. The results were observed after 2 weeks.

3.3.9.6 Effect of Growth Regulators on Shoot Proliferation from Seed Sprouts

Seeds were tested for various concentrations of BAP (1.1 to 17.6 µM) and Kn (1.16 to 9.3 µM) to study their effect on multiplication rate. Five replicates were used for each treatment and proliferation of shoots was observed after 30 and 45 days and percentage proliferation was recorded.

3.3.9.7 Callus Induction

The seeds were properly surface sterilized and transferred onto MS medium supplemented with various PGR combinations and the medium without PGRs served as a control. The medium was gelled with 0.8% agar and supplied with 3% sucrose unless otherwise mentioned and the details of combinations employed for callus induction are as follows:

1. MS + 2,4-D (1.13-18.12 µM)
2. MS + NAA (1.34-24.16 µM)
3. MS + BAP (1.1-17.6 µM)
4. MS + 2,4-D (1.13-18.12 µM) + NAA (1.34-24.16 µM)
5. MS + 2,4-D (1.13-18.12 µM) + BAP (1.1-17.6 µM)

3.3.9.8 Effect of Sucrose on Relative Growth Rate (RGR)

The effect of sucrose on relative growth rate was studied using 3.0 gm of fresh callus tissue and various concentrations of sucrose ranging from 1 to 5%. Observations were recorded after every 4 weeks.

3.3.9.9 Effect of MS Strength on RGR

To test the effect of strength of MS medium on RGR, the following three strengths of MS were tried: MS full strength, ½ MS and ¼ MS and results were recorded.

3.3.9.10 Effect of GA₃ on RGR

GA₃ has role in improving the proliferation of callus in bamboos and therefore various concentrations of GA₃ (1.1 to 2.8 µM) were used in the present experiments to standardize the best concentration for the growth.

3.3.9.11 Effect of Myoinositol on RGR

Myoinositol is helpful in cell division activity and therefore can effects the growth rate of callus. Inositol at a concentration of 100 to 500 mg/l was tested for its effect on the growth of callus and data was recorded after fixed time intervals.

3.3.9.12 Starch Test for Seeds

Longitudinal hand sections of seeds were stained with weak solutions of Iodine (I₂) for 20 minutes in a Petriplate (3.0 cm) for observing the presence of starch. I₂ solution was prepared by dissolving 0.3 g of I₂ crystals and 1.5 g of potassium iodide (KI) in water and final volume was made upto 100 ml. The presence of starch was confirmed by appearance of dark blue-black colour.

3.3.10 Evaluation of *In Vitro* Plants and 6 Months Old *Ex Vitro* Hardened Plants

A systemic study on the changes occurring at the physiological and biochemical levels to the plants during *ex vitro* hardening was carried out and details are outlined as follow:

3.3.10.1 Stomatal Studies

Stomatal density was compared in the 6 weeks old *in vitro* plants and hardened field established plants. Leaf segments of fully expanded third leaf from the top of each branch were taken for the study around 10 am in the morning. For determination of stomatal density, the method as described by Bag et al. (2000) was used. For the determination of stomatal frequency, impressions of both the adaxial and abaxial surfaces of leaf were taken on white glue. Stomatal frequency was counted following random observations under light microscope and final frequency was calculated for both sets of plants. SEM analysis of the leaves was performed using method of Saha et al. (2011) on a Scanning Electron Microscope. In this method, samples were prepared by removing all the impurities like dirt and agar sticking to them. Samples were then fixed in 2-5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.0) for 1 hour followed by washing with 0.001 M of phosphate buffer containing 7% sucrose till the traces of glutaraldehyde were removed. Dehydration was done by an organic solvent in a series for which samples were placed in 30%, 50%, 70%, 80% acetone for 15 minutes each; 90% acetone for 20 minutes and finally in 100% acetone for 30 minutes. Further, samples were dehydrated in acetone: amyloacetate for 15 minutes followed by only amyloacetate for 15 minutes. They were then fixed on blocks and viewed under SEM at different magnifications.

3.3.10.2 Leaf Area Index

Leaf area index was studied by millimeter graph paper method using a millimeter graph paper (Pandey and Singh, 2011). Estimate of leaf area was obtained by the equation:

$$\text{Leaf area (cm}^2\text{)} = x/y$$

Where x = weight in (g) of area covered by the leaf outline on a millimeter graph paper

y = weight of 1 cm² on same graph paper

3.3.10.3 Relative Water Content (RWC)

Relative water content was determined according to the method of Perl-Treves and Galum, (1991). From each leaf, 9 discs were cut and fresh weight was measured. Leaf discs were immersed in distilled water for 20-24 hours and their hydrated weights were also measured. The samples were then dried overnight in an oven maintained at 70°C and their dry weights were measured.

RWC was calculated by the formula:

$$100 \times \frac{\text{actual weight-dry weight}}{\text{hydrated weight -dry weight}}$$

Water Saturation Deficit (WSD) was calculated by the formula:

$$\text{WSD} = 100 - \text{RWC}.$$

3.3.10.4 Electrolyte Leakage

For estimating cell membrane stability, electrolyte leakage was measured using an electrical conductivity meter by the method described by Wright and Simon, (1973). Eight leaf discs were cut, rinsed and immersed in distilled water for 22 hours. Initial readings were taken in $\mu\text{S}\cdot\text{cm}^{-1}$ before autoclaving and again reading was taken using conductivity meter (Model 510^{PC}, Cyberscan, China). Results were expressed as percentage of electrolyte leakage.

$$\text{Electrolyte leakage \%} = 100 \times \text{EL before boiling} / \text{EL after boiling}$$

3.3.10.5 Lignin Estimation

About 2 cm basal portions of young culms weighing 50 mg were taken and lignin was estimated by using acetyl bromide method (Iiyama and Wallis, 1990). The samples were placed at 80 °C for 1.5 hour with 3 ml of 80% (v/v) ethanol in test tubes. Three successive extractions were done with this solution before extracting the samples. Segments were dried for 2 days in an oven at 50°C followed by their digestion in 2.6 ml of solution of 25% (v/v) acetyl bromide in acetic acid containing 2.7% (v/v) perchloric acid. 100 μl of each sample was taken after 1 hour and added to 580 μl of a solution of 17.24 % (v/v) 2 N sodium hydroxide (NaOH) and 82.76% (v/v) glacial acetic acid (GAA) and 20 μl of 7.5 mol/l hydroxylamine HCl to ensure termination of reaction. Volume was corrected with 2 ml of acetic acid. Absorbance was measured at A_{280} and lignin content measured in g fr. wt. as:

$$X = (Y - 0.0009) / 20.09$$

Where X is the = Concentration of lignin (mg/ml)

Y = Optical density

0.0009 = Mean intercept value

20.09 = Mean extinction co-efficient.

Similarly, biochemical changes were discerned and data collected during the acclimatization and the parameters studied included:

3.3.10.6 Chlorophyll Content

The green epidermis from culms of *in vitro* and *ex vitro* grown plants was peeled and grounded to a fine powder in a pestle and mortar. Epidermis powder 40 mg fresh weight (fr. wt.) was added to sample vial containing 25 ml each of either of the three solvents namely Acetone, Dimethylformamide (DMF) or Dimethylsulfoxide (DMSO) and the chlorophyll was extracted using an ultrasonicator for 3 minutes using method of Chang et al. (1998). The chlorophyll solutions were analysed with an ultraviolet (UV)-Vis Spectrophotometer (Biotek Synergy, HT). Finally chlorophyll content in mg/g fr. wt. was calculated by using the equation:

$$\text{Chl a: } [12.7 \times \text{OD}_{663} - 2.60 \text{OD}_{645}] \times \text{factor}$$

$$\text{Chl b: } [22.7 \times \text{OD}_{645} - 4.68 \text{OD}_{663}] \times \text{factor}$$

3.3.10.7 Total Soluble Sugars (TSS)

TSS was calculated using the method of Mc Cready et al. (1950). 100 mg of fresh leaf tissue was homogenized in 80 % ethanol and centrifuged at 12000 rpm for 3 minutes. 200 µl aliquot of tissue extract was pipetted out in 10 ml test tube and volume was made upto 1 ml by adding 800 µl of distilled water. To this added 4 ml of freshly prepared anthrone reagent and allowed it to run down the sides of test tube and vortexed it for proper mixing. Anthrone reagent was prepared by dissolving 200 mg anthrone in 100 ml of concentrated sulphuric acid (H₂SO₄). It yielded a yellowish green coloured reagent. The tubes with reaction mixture were placed on boiling water bath for 8 minutes and allowed to cool rapidly under running tap water to yield a dark green solution. Supernatant was taken for estimation of TSS in anthrone reagent. Glucose content was measured at A₆₃₀ with the help of standard graph and obtained final volume by x 0.9 factor.

3.3.10.8 Estimation of Starch

Starch was measured as liberated glucose using anthrone reagent after hydrolyzing the extracted tissue with perchloric acid following method of Adams et al. (1980). Homogenised 100 mg of fresh tissue in 80% hot ethanol followed by centrifugation at 12000 rpm for 3 minutes. Discarded the supernatant, added 5 ml of distilled water and 6.5 ml of 52% perchloric acid to the residue and allowed it to react for 20 minutes at 0°C with gentle shaking. Centrifuged at 12000 rpm for 3 minutes and used 200 µl aliquots of supernatant for estimation of starch content with anthrone reagent.

3.3.10.9 Proline Estimation

Proline is a compatible osmolyte and generally produced during water and salt stress. In order to compare the levels of proline in the *in vitro* and *ex vitro* grown plants, proline was

estimated using the method of Bates, (1973). The leaf samples were grounded using liquid nitrogen followed by addition of sulfosalicylic acid (10 ml) to 0.5 g of the plant tissue in a test tube. Vortexed it for 10 seconds and left it for 5 minutes. After 2nd vortexing, the suspension was filtered. 2 ml of extract was placed in a 15 ml test tube and treated with ninhydrin acid reagent (2 ml sample and 1.25 g ninhydrin) in 6M phosphoric acid (20 ml) with gentle heating. After that 2 ml of glacial acetic acid (GAA) was added and each sample was vortexed for 10 seconds. Sealed test tubes were heated for 1 hour in a water bath at 95-98 °C. After quickly cooling in an ice cold water bath, 4 ml toluene was added to each sample. Test tubes were sealed again, vortexed for 15 seconds and left at room temperature until upper toluene layer with proline was clearly separated from the lower water layer. Final results were expressed as μ mol proline/g of fresh or frozen plant sample. Proline was quantified in the upper phase using ninhydrin acid reagent. The chromophore containing proline was extracted in 4 ml of toluene and measured spectrophotometrically at 520 nm. L-proline was used as a standard.

3.3.11 Anatomical Studies

Samples like leaf, culms and roots were used for microtomy. Likewise, for ascertaining different morphogenetic stages, the calli were also used for anatomical studies.

i. Fixing and Killing of Material

The material was fixed in a freshly prepared fixative FAA (Formalin: Acetic acid: 50% Ethanol:: 5: 5: 90) and subsequently in 70% ethanol until further use, after which the tissue was dehydrated in TBA (t-butyl alcohol) series as given in [Table 3.3.11](#)

Table 3.3.11 Dehydration of tissue in different TBA series

Step No.	Ethanol (ml)	TBA (ml)	Water (ml)
1	30	20	50
2	50	20	30
3	50	35	15
4	45	55	-
5	25	75	-
6	-	100	

The material was kept in each grade for 3-4 hrs except in Step No.3 where it was kept overnight.

ii. Waxing

For waxing, the tissue in TBA was kept in an oven preset at 60°C and paraffin wax flakes were added after every 15-20 min. The whole process was carried out till there was no smell of TBA left in the samples indicating complete waxing. The blocks were made and 12 µm thick sections were cut using a microtome and stretched on the glass slides. De-waxing was done in the following grades and sections stained in safranin and fast green.

1. 75 ml Xylol: 25 ml Ethanol
2. 50 ml Xylol: 50 ml Ethanol
3. 25 ml Xylol: 75 ml Ethanol
4. 100 ml Ethanol
5. 25 ml Water: 75 ml Ethanol
6. 50 ml Water: 50 ml Ethanol
7. 75 ml Water: 25 ml Ethanol
8. Safranin (6-24 hours)
9. 75 ml Water: 25 ml Ethanol
10. 50 ml Water: 50 ml Ethanol
11. 25 ml Water: 75 ml Ethanol
12. Ethanol -1
13. Ethanol - 2
14. Clove oil 25% in Ethanol
15. Clove oil 50% in Ethanol
16. Fast green (prepared in 50 % clove oil)
17. Clove oil 50% in Xylol
18. Clove oil 25% in Xylol
19. Xylol-1 (30 minutes)
20. Xylol-2 (30 minutes)
21. Mounted the slides in D.P.X. mountant

Sections were examined under the light microscope and photographed by means of an automatic photomicrography system.

3.4 Genomic DNA Isolation and PCR Amplification

3.4.1 Protocol for DNA Isolation: In a bid to ascertain the genetic fidelity of tissue culture raised plants, random samples of leaves were taken from the mother plant and 6 month old hardened plants growing in the greenhouse for isolation of DNA. DNA was extracted from fresh fully expanded leaves using cetyl trimethylammonium bromide (CTAB) method of

Doyle and Doyle, (1990). Leaf tissue (3-4 gm) was crushed in liquid nitrogen and 100 mg polyvinyl pyrrolidone (PVP) was added. Pre-heated extraction buffer was added (10-15 ml), thawed and transferred to centrifuge/oakridge tubes. The samples were incubated at 65 °C for 1 hour in a water bath. Proper mixing was done after half an hour by inversions. Phenol: chloroform: isoamyl alcohol::: 25:24:1 were then added to it at room temperature. Mixed gently by inversions and care was taken to prevent foaming. Samples were then centrifuged at 13000 rpm at 25 °C for 15 to 20 minutes. The upper aqueous phase was transferred using wide bore tips (avoid contamination from the interphase) and 0.6 ml volume of isopropanol was added to the aqueous phase. After mixing by gentle inversions, samples were centrifuged at 10000 rpm for 30 minutes at 4°C. Supernatant was discarded and pellet was spooled out. The pellet was washed with 70% chilled ethanol and centrifuged at 10000 rpm for 10 minutes at room temperature. The dry pellet was dissolved in 200 µl of Tris-EDTA (TE) buffer.

Extraction buffer (100 ml) consisted of:

- a. 1M Tris-HCl Trizma base-10 ml
- b. Na-EDTA- 4 ml
- c. Sodium Chloride (NaCl)-35 ml
- d. CTAB-20 ml
- e. A.DH₂O-30.8 ml
- f. β-mercaptoethanol-0.2 ml

3.4.2 Quantification of DNA:

- a) Calibrated the instrument (UV/ VIS spectrophotometer).
- b) Took 5 ml of the DNA sample in a 1ml quartz cuvette. Made up the volume to 1 ml with autoclaved distilled water (control tube contained 5 ml of TE in 1 ml of water).
- c) Measured absorbance of the solution at 260 nm and 280 nm.
- d) Calculated the 260/280 ratio of absorbance. A pure DNA sample should exhibit $A_{260}/A_{280} = 1.8$. If ratio is > 1.8 , the DNA is likely to be contaminated by RNA and if it is < 1.8 , it is likely to be contaminated by proteins and phenol.
- e) Calculated the DNA concentration in the solution by using the relationship for double stranded DNA as 1 O.D. at 260 nm = 50 mg/ml.

3.4.3 Optimization of PCR Parameters:

Thirty RAPD primers and twenty ISSR primers at a concentration of 1 to 1.5 µM / reaction were scanned in the present study. PCR amplifications were carried out in total volume of 25 µl containing 20 ng of genomic DNA. The reaction buffer for RAPD consisted of 2.5 µl *Taq* buffer, 0.15 to 0.2 µl dNTP, 1.5 µl primer, 0.17 µl *Taq* Pol, 18.13 µl DW and 2 µl (20-25

ng) DNA. For RAPD, PCR amplification was carried out in Bio Rad I cycler from Bio Rad Laboratories (India) Pvt. at an initial denaturation at 94 °C for 5 minutes, followed by 45 cycles of 1 minute denaturation at 94 °C, 1 minute annealing at 37 °C and 2 minutes extension at 72 °C, with a final extension at 72 °C for 7 minutes. The reaction buffer for ISSR consisted of 2.5 µl *Taq* buffer, 1 µl MgCl₂, 0.15 µl dNTP, 1.5 µl primer, 0.17 µl *Taq* Pol, 17.68 DW and 2 µl (20-25 ng) DNA. The ISSR was programmed for initial DNA denaturation at 94 °C for 4 minutes, followed by 44 cycles of 1 minute denaturation at 94 °C, 1 minute annealing (temperature specific to the primer) and 1 minute extension at 72 °C with a final extension at 72 °C for 7 minutes. The amplified fragments were electrophoresed in 1.8% agarose gel matrix in an electrophoresis system (Bio Rad Subsell 96) for atleast 2 hours or until the bands were clearly separated and visible. The constant power supply was maintained at 65V with the help of Powerpac 300 (Bio Rad Laboratories, India, Pvt. Ltd). Gel photographs were analysed through Gel Doc system (Molecular Imager[®], Gel Doc[™] XR Imaging System).

3.4.4 Scoring of Bands

For each primer, bands were scored manually for the presence (1) or absence (0) of the amplified products. Number of bands produced and length were analysed. The size of the amplified products was estimated using a 100 bp ladder and 500 bp ladders (Bangalore Genei Pvt. Ltd, Bangalore, India).

3.5 Field Performance of *In Vitro* Raised Plants

Field performance of *in vitro* raised plants under the field conditions was recorded by studying different plant parameters like height, number of shoots, number of leaves, number of axillary shoots per clump and number of internodes per shoot and the data was recorded for the six months under field conditions.

3.6 Statistical Analysis

All the investigations were based on Complete Randomized Block Design (CRBD) with minimum of 3 replicates. The data shown represent the Mean ± SD. Significance of treatment was determined by one way ANOVA and the means were compared with Duncan's test at a significance of $P \leq 0.05$ using STATISTICA, release 7, Statsoft Wipro, Bangalore, India.

4. Results

Objective 1: To develop and standardize an efficient, reliable and reproducible protocol for mass propagation of *Bambusa bambos*, *Bambusa balcooa* and *Dendrocalamus membranaceus* under *in vitro* conditions.

4.1 Micropropagation studies in *Dendrocalamus membranaceus* Munro

4.1.1 Selection of Elite Mother Plant

Prior selection of elite mother plant holds great importance while carrying out micropropagation studies. In the present investigation, a number of parameters like a) height of the culm b) girth of the culm at third internode from the bottom c) number of culms per clump and d) length of internode were taken into consideration while selecting the mother plant. The explants were taken from precocious branches of 1 year old field grown plant.

4.1.2 Choice of Explants

Various explants like nodal explants, apical buds, leaf bases and seeds were used for initiating aseptic cultures. Explants were washed under running tap water for 25-30 minutes for removal of dust particles and microbes adhered to the surface. The explants were then cleaned with the liquid detergent with the help of a sable hair brush and then washed with autoclaved distilled water to remove the traces of the detergent. The explants were further treated with a suitable fungicide like bavistin (0.1 to 1%) and antibacterial agents like streptomycin sulphate (0.02 to 1%) and tetracycline (0.01 to 0.1%) for another 25-30 minutes. Bavistin at 0.25%, streptomycin sulphate and tetracycline at 0.02% each were found to be the most effective treatments. The explants were further subjected to treatments with sterilants in a laminar air flow cabinet. They were washed with 70% alcohol for 1 minute followed by treatments with 15 % NaOCl for 8 minutes and HgCl₂ (0.1% w/v) for 8 minutes for nodal explants, 6 minutes for apical buds, 5 minutes for leaf bases and 10 minutes for seeds.

Apical buds taken from secondary and tertiary branches of elite mother plant turned necrotic after 1 week of inoculation. Likewise, no response was observed when leaf bases were inoculated onto MS medium on any of the combinations of growth regulators tried. Only the nodal explants taken from secondary branches and seeds gave the favourable response for initiating aseptic cultures.

4.1.3 Nodal Explants

4.1.3.1 Seasonal Effect on Culture Initiation

The maturity status of the explants varied with season and directly affected the response of explant to culture initiation. The best period for initiating aseptic cultures was spring (February-April) when maximum bud break (90%) was achieved followed by rainy season depicting 80% bud sprouting on basal MS medium (Figure 4.1.3.1). However, during rainy season (July-August), maximum contamination (95%) was observed. Hence, rainy season was not a preferred season as it had a direct influence on contamination rate and survival percentage of explants.

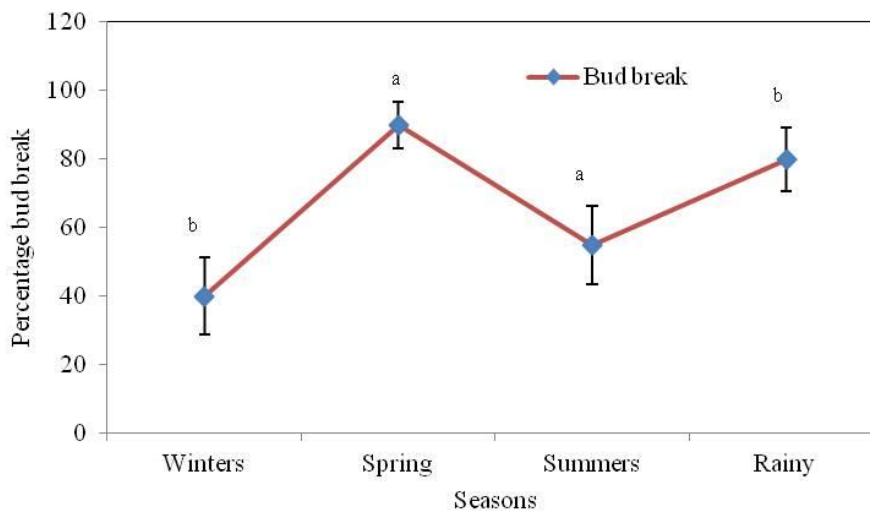


Figure 4.1.3.1. Variation in bud-break during different seasons (year 2011)

4.1.3.2 Effect of Explant Size

For initiation of aseptic cultures, explant size markedly influenced the bud break. The larger explants (25 mm) took the least number of days for sprouting which started after 14 days of culture (Figure 4.1.3.2). The small sized (5 and 10 mm) explants took much longer time i.e. 24 days for sprouting and that too in a few cultures only. The larger explants probably contained more nutrient reserves and plant growth regulators (PGR's) to sustain themselves during the initiation phase of cultures.

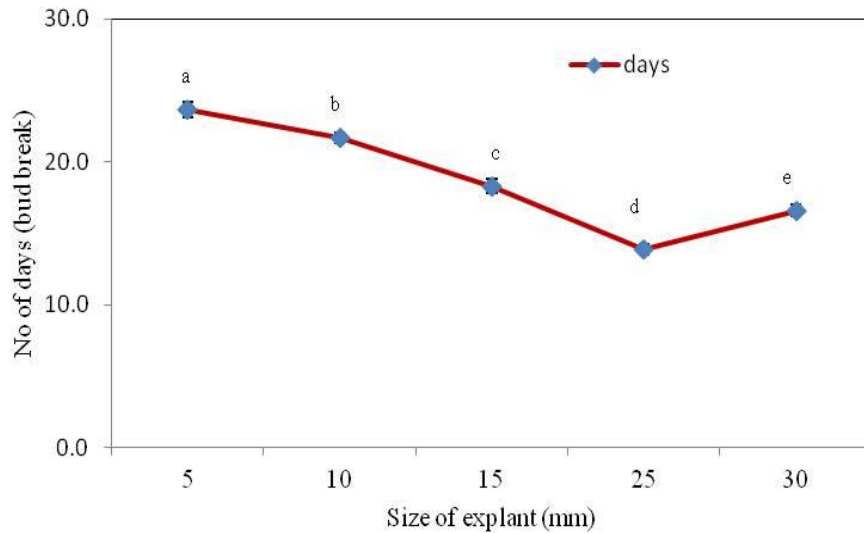


Figure 4.1.3.2. Effect of explant size on bud break.

4.1.3.3 Effect of Plant Growth Regulators on Shoot Multiplication

For inducing sprouting, nodal explants were inoculated on basal MS medium without any growth regulator where initial bud break occurred after 7-10 days (Figure 4.1.3.3 a). Thereafter, the sprouted buds were transferred onto MS medium containing different concentrations and combinations of cytokinins like BAP, Kn and adenine sulphate for inducing axillary shoot proliferation (Figure 4.1.3.3 b). The number of sprouted buds at each node was treated as individual shoot during present investigation. Out of the various cytokinins tested, multiple shoots were formed on BAP (4.4 μ M) supplemented medium where 7.50 ± 0.8 shoots were formed after 15 days (Figure 4.1.3.3 c). However, the best shoot proliferation occurred when 4.4 μ M of BAP was used in conjunction with 1.16 μ M of Kn, thereby, showing a definite synergism between the two. On this medium multiple shoot proliferation occurred after 1 week forming a few shoots after 20 days (Figure 4.1.3.3 d) which multiplied further forming 13.40 ± 1.5 shoots per explant, with a shoot length of 2.92 ± 0.31 cm and number of leaves being 15.50 ± 0.97 after 30 days (Figure 4.1.3.3 e). Higher doses of BAP (13.2 μ M) induced thin leaf like shoots, which did not develop further. Addition of Kn alone was not effective in shoot proliferation. The effect of different cytokinins on shoot proliferation is depicted in Table 4.1.3.3. Once the clusters of shoots were formed, small clumps of 3-4 shoots were excised and transferred onto fresh multiplication medium every 4 weeks to avoid the browning of cultures and for achieving continuous shoot proliferation. The shoots could be multiplied consistently for 15 passages without any decline in the growth. The number of propagules used for recurrent

multiplication of shoots was a critical factor during present study. Shoot clumps each having 3 shoots rather than single shoot were found to be most effective for further multiplication of shoots. Single shoot if excised from the sprouted bud and sub-cultured for multiplication failed to survive. Likewise, use of more number of shoots per propagule (6, 9) failed to give good multiplication rate.



Figure 4.1.3.3 a. Axillary bud break on basal MS medium after 10 days of inoculation. **Figure 4.1.3.3 b.** Excised shoot clumps of 3-4 shoots on BAP (4.4 μ M) **Figure 4.1.3.3 c.** Multiple shoots on BAP (4.4 μ M) after 15 days. **Figure 4.1.3.3 d.** Multiple shoot proliferation on BAP (4.4 μ M) and Kn (1.16 μ M) after 20 days. **Figure 4.1.3.3 e.** Shoot multiplication after 30 days on the same medium.

Once the best combination of cytokinins for shoot multiplication was found, the cytokinin-supplemented medium was tested with the addition of different auxins such as IBA, IAA and NAA. It was observed that the addition of auxins into multiplication medium considerably reduced the proliferation rate. Hence, their addition to the multiplication medium was discontinued.

Table 4.1.3.3: Effects of BAP and Kn Concentrations on Proliferation of Shoots

BAP + Kn (μM)	No of shoots	Shoot Length (cm)	Total no of leaves
0.0+ 0.0	2.80 ^f ± 1.22	1.20 ^g ± 0.25	4.20 ^h ± 0.78
0.0+ 4.6	2.60 ^{fg} ± 0.69	1.10 ^g ± 0.17	2.40 ^{li} ± 0.5
0.0+ 1.16	2.50 ^{fg} ± 0.52	1.17 ^g ± 0.23	3.50 ^h ± 0.52
0.0+ 2.2	2.50 ^{fg} ± 0.70	1.10 ^g ± 0.12	2.50 ⁱ ± 0.52
4.4+ 0.0	7.50 ^c ± 0.84	1.60 ^{ef} ± 0.61	9.90 ^c ± 0.73
4.4+ 0.46	9.30 ^b ± 1.15	2.20 ^{bc} ± 0.71	12.10 ^b ± 0.73
4.4+ 1.16	13.40 ^a ± 1.57	2.92 ^a ± 0.31	15.50 ^a ± 0.97
4.4+ 2.2	8.80 ^b ± 1.39	2.45 ^b ± 0.43	9.10 ^{cd} ± 1.52
8.8+ 0.0	5.60 ^d ± 0.96	2.00 ^{cd} ± 0.40	7.30 ^f ± 1.15
8.8+ 0.46	6.20 ^d ± 0.78	2.45 ^b ± 0.43	7.70 ^{ef} ± 1.15
8.8+ 1.16	7.20 ^c ± 0.91	2.35 ^{bc} ± 0.33	8.85 ^{de} ± 1.08
8.8+ 2.2	3.80 ^e ± 0.91	2.35 ^{bc} ± 0.24	7.30 ^f ± 0.67
13.2+ 0.0	2.20 ^{fg} ± 0.78	1.65 ^{def} ± 0.41	5.80 ^g ± 0.91
13.2+ 0.46	2.50 ^{fg} ± 0.70	1.70 ^{def} ± 0.34	7.30 ^f ± 0.67
13.2+1.16	2.70 ^f ± 0.94	1.80 ^{de} ± 0.25	7.60 ^f ± 1.26
13.2+2.2	1.70 ^g ± 0.82	1.40 ^{fg} ± 0.39	5.20 ^g ± 0.78

Mean value of 5 replicates. Different *uppercase* letters within the *columns* indicate significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).

4.1.3.4 Effect of Sucrose Concentrations on Shoot Multiplication

To study the effect of different concentrations of sucrose on shoot multiplication, three to four shoots/clump were cultured on MS medium containing BAP (4.4 μM) and Kn (1.16 μM) supplemented with different concentrations of sucrose (0.5 to 3%). Out of different concentrations tested, 2% sucrose was found to be most suitable carbon source where 13.40 ± 1.5 shoots were formed after 4 weeks (Figure 4.1.3.4). Moreover, the replacement of sugar with less expensive table sugar did not affect multiplication rate significantly, instead it reduced cost of production considerably. Hence, table sugar was preferred over sucrose for carrying out micropropagation studies presently.

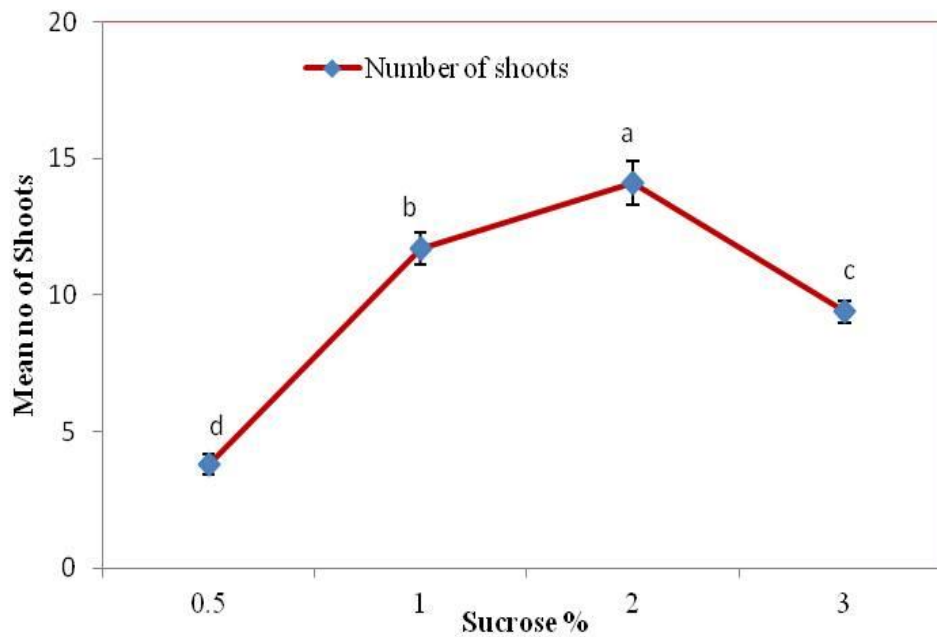


Figure 4.1.3.4. Effect of sucrose concentrations on shoot multiplication

4.1.4 Rooting of Microshoots

Rooting is a major bottleneck while carrying out *in vitro* multiplication of bamboos. In the present study it was observed that rooting was more effectively induced when clusters of shoots rather than individual shoots were used. The clumps of 3-4 shoots were cultured onto MS medium and modified MS with major salts reduced to half strength (1/2 MS) and one-fourth strength (1/4th MS). These media were fortified with different growth regulators gelled with 0.8 % agar or 0.25% phytigel. Since in all the media combinations involving 1/2 MS, the results were better than full strength MS or 1/4th strength MS, all the rooting experiments were carried out on 1/2 MS medium. Among the three auxins i.e. NAA, IBA and IAA tested, 5.37 μM of NAA when used in conjunction with 4.4 μM of BAP was found to be most effective for root induction with maximum root number (6.00 ± 1.00 roots per propagule) and an average root length of 6.68 ± 0.72 cm achieved within 30-45 days (Figure 4.1.4 a) Within 60 days, well developed root system was seen in almost 65% cultures (Figure 4.1.4 b). With further increase in concentration of NAA (10.74 μM), a marked reduction in rooting number was observed. The results of root induction achieved on different concentrations of NAA and BAP are given in Table 4.1.4. No root formation was observed when shoots were cultured on MS medium containing IAA or IBA either alone or in conjunction with BAP.

Table 4.1.4 Effect of NAA and BAP on rooting in *D. membranaceus*

NAA/BAP (μM)	No. of Roots	Root Length (cm)	Rooting %
0.0/2.2	0.0 ^d \pm 0.00	0.0 ⁱ \pm 0.00	0
0.0/4.4	0.0 ^d \pm 0.00	0.0 ⁱ \pm 0.00	0
0.0/8.8	0.0 ^d \pm 0.00	0.0 ⁱ \pm 0.00	0
0.53/0.0	0.0 ^d \pm 0.00	0.0 ⁱ \pm 0.00	0
0.53/2.2	0.0 ^d \pm 0.00	0.0 ⁱ \pm 0.00	0
0.53/4.4	0.5 ^c \pm 0.52	5.3 ^{de} \pm 0.67	10
0.53/8.8	1.3 ^b \pm 0.48	5.0 ^{ef} \pm 0.8	21
5.37/0.0	1.4 ^b \pm 0.51	7.1 ^a \pm 0.73	30
5.37/2.2	1.4 ^b \pm 0.51	7.2 ^a \pm 0.82	43
5.37/4.4	6.0 ^a \pm 1.05	6.6 ^{ab} \pm 0.72	65
5.37/8.8	1.7 ^b \pm 0.48	6.3 ^{bc} \pm 0.70	50
10.74/0.0	1.6 ^b \pm 0.51	5.8 ^{cd} \pm 0.86	42
10.74/2.2	1.4 ^b \pm 0.51	4.7 ^{fg} \pm 0.63	30
10.74/4.4	1.4 ^b \pm 0.51	4.3 ^g \pm 0.94	23
10.74/8.8	1.3 ^b \pm 0.48	3.6 ^h \pm 0.4	20

Mean value of 5 replicates. Different *uppercase* letters within the *columns* indicate significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).



Figure 4.1.4 a. Root formation on a 1/2 strength MS medium fortified with 5.37 μM of NAA and 4.4 μM of BAP after 45 days. **Figure 4.1.4 b.** Rooted plantlets after 60 days.

4.1.5 Hardening and Acclimatization

Plantlets were taken out of flasks and washed with luke warm water to remove any agar sticking to them. These were then transferred to plastic pots containing river bed sand and covered with perforated plastic covers/ jars to maintain high internal humidity and were kept in a growth room. After 14 days, the plantlets were transferred to potting mixture containing sand: soil: farmyard manure (1:1:1) and shifted to green house (Figure 4.1.5 a). In the green house, the controlled atmospheric conditions of low irradiance, high RH of 60-80%, temperature not exceeding 28 °C were provided to promote the plant growth (Figure 4.1.5 b). The foliage was sprayed with Hoagland solution after every 7 days. Hardened plants were finally transplanted in the field in the rainy season to pits (2ft×2ft×2ft) at a plant to plant and row to row distance of 6 meters and were successfully established under field conditions. (Figure 4.1.5 c and d). Plants showed well developed root and shoot systems and all the plants are thriving very well in field conditions with no phenotypic variations observed when compared to the mother plant.



Figure 4.1.5 a. Acclimatized plants of *D. membranaceus* in green house on mixture of Soil: Sand: FYM :: (1:1:1). **Figure 4.1.5 b.** Hardened plants after 6 months.

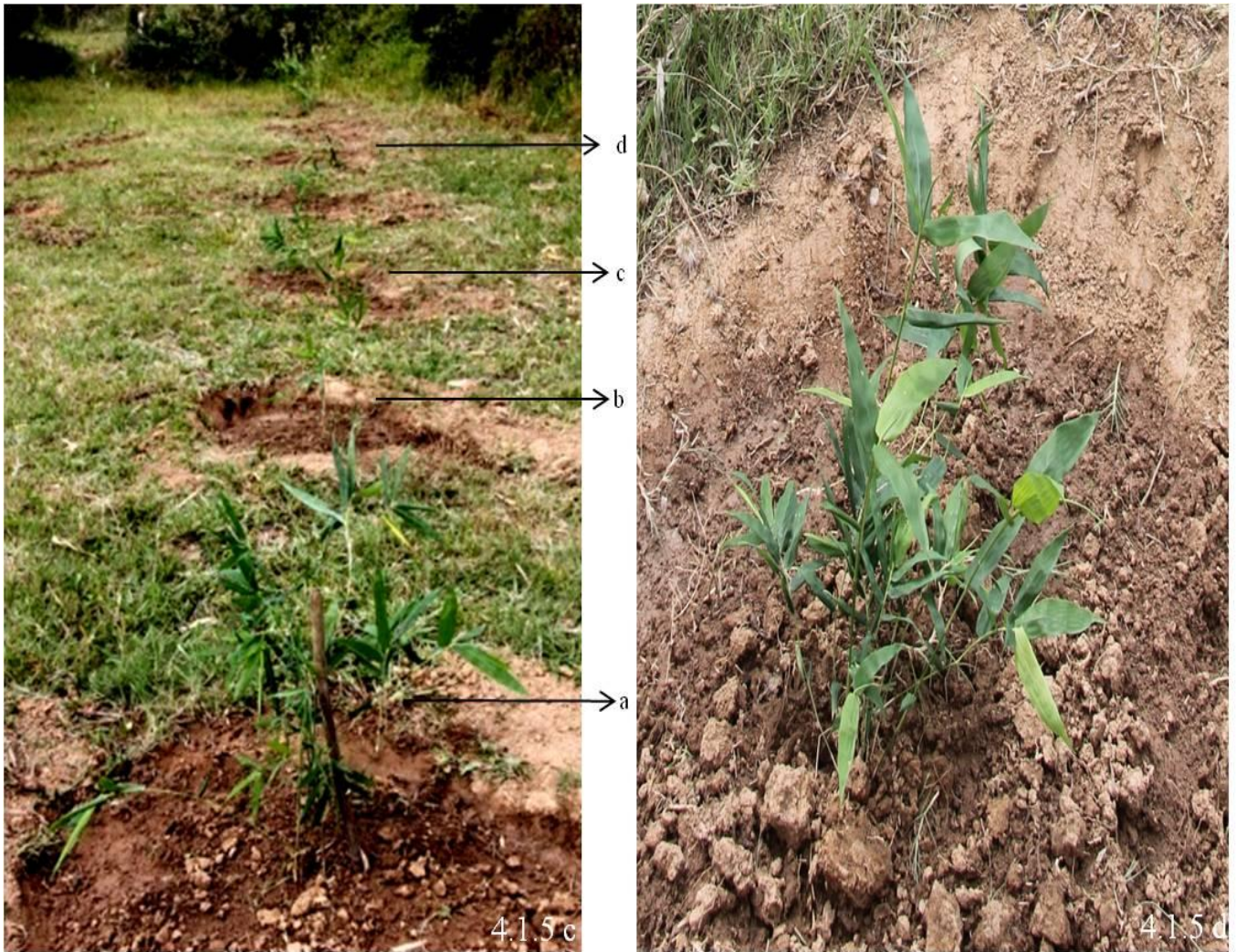


Figure 4.1.5 c. Hardened plants (a-d) growing in pits (2 ft x 2ft x 2 ft) at a plant to plant and row to row distance of 6m. **Figure 4.1.5 d.** Hardened plant of *D. membranaceus* under field conditions after 6 month of transfer.

4.1.6 Callus Induction and Organogenetic Differentiation

4.1.6.1 Induction of Callus

Internodal segments (0.5-1cm) were excised from *in vitro* raised shoots and were transferred onto MS medium supplemented with different concentrations of auxins and cytokinins for callus induction. Incorporation of 2, 4-D in the medium was found to be essential for callus initiation. Addition of NAA (5.37 μM) to 2, 4-D (4.53 μM) supplemented medium further accelerated the callus growth when incubated in dark. Callusing started at the cut ends of the explant after 15 days (**Figure 4.1.6.1 a**). During callus induction, swellings were observed at the cut ends of the explant representing divisional activity within the tissue which ultimately resulted in the rupturing of epidermis and exposure of callus tissue from within. After 6 weeks the whole explant turned into a mass of yellow and compact callus (**Figure 4.1.6.1 b**)

which became nodular after 8 weeks (Figure 4.1.6.1 c). The nodular and compact callus was capable of sustained growth on subculturing on the same medium. Occasionally, mucilaginous callus was also obtained but only nodular and compact callus was retained for further experimentation because mucilaginous callus was found to be unresponsive to regeneration. Effects of various PGRs on callus induction are given in the Table 4.1.6.1.

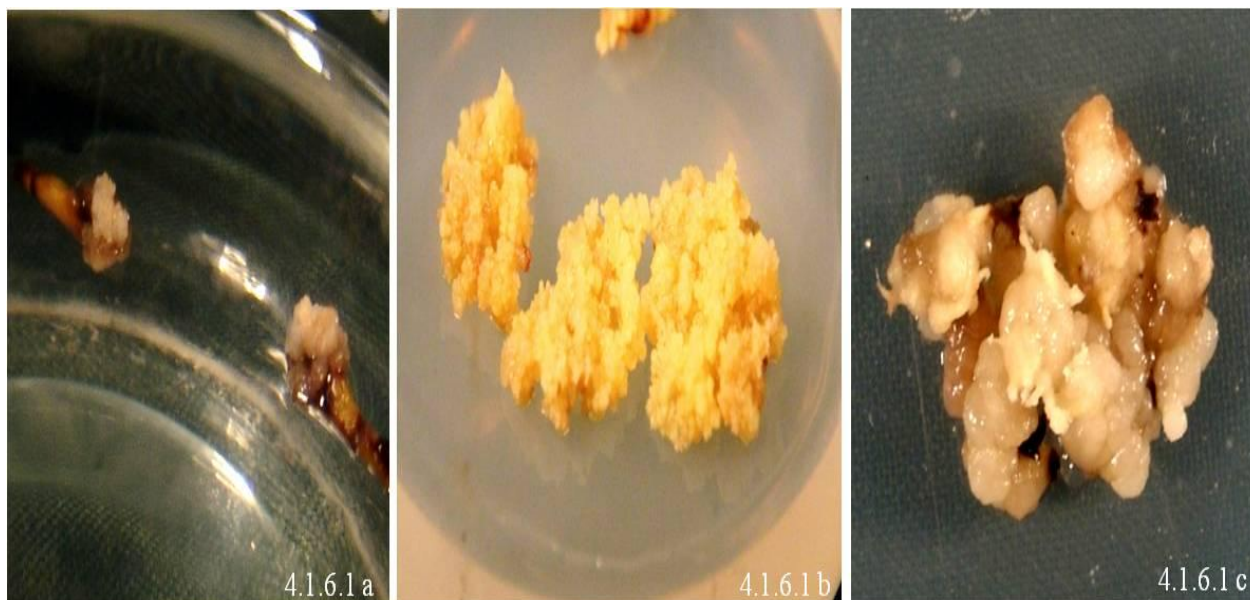


Figure 4.1.6.1 a. Initiation of callus on 4.53 μM of 2,4-D and 5.37 μM of NAA after 15 days **Figure 4.1.6.1 b.** Proliferation of callus after 6 weeks. **Figure 4.1.6.1 c.** Callus turned nodular after 8 weeks on the same media.

Table 4.1.6.1 Effect of Media Composition on Callus Induction from Internodal Segments

S.No.	Media Composition	Concentration (μM)	% Callus Induction	Days Required
1	-	-	0	-
2	MS+ 2,4-D	0.45	20	30
3	MS+ 2,4-D	2.26	50	27
4	MS+ 2,4-D	4.53	70	25
5	MS+NAA	2.68	0	-
6	MS+NAA	5.37	0	-
7	MS+ 2,4-D+NAA	4.53+5.37	80	15
8	MS+ 2,4-D+NAA	9.06+ 10.74	60	23

4.1.6.2 Caulogenesis

For caulogenesis, nodular callus was shifted under cool, white 40 W fluorescent lamps with irradiation at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) with alternating 16/8 h light: dark cycle. Shoot bud induction occurred after 10 days exposure to light on the medium containing $4.53 \mu\text{M}$ of 2, 4-D and $5.37 \mu\text{M}$ of NAA (Figure 4.1.6.2 a) but the buds did not grow further. However, when shifted to MS medium supplemented with low concentration of BAP ($4.4 \mu\text{M}$) in combination with Kn ($1.15 \mu\text{M}$), shoot buds elongated and leaves were formed after 30 days as shown in Figure 4.1.6.2 b. These shoots further elongated on the same medium as seen after 45 days (Figure 4.1.6.2 c). The frequency of shoot bud differentiation was very low being 15% only. Addition of any other cytokinin like TDZ and 2-ip did not prove effective for inducing organogenesis, instead, they resulted in the browning of callus. Incorporation of any additional auxins (NAA, IBA, IAA) also turned the callus necrotic.

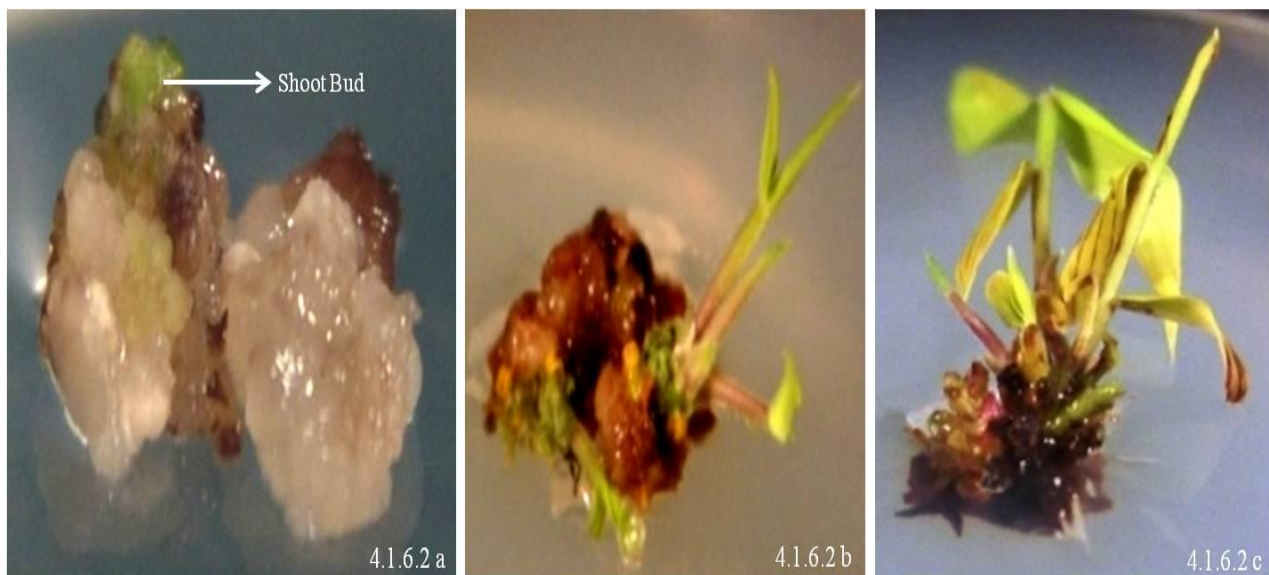


Figure 4.1.6.2 a. Shoot bud differentiation from callus when shifted to light on $4.53 \mu\text{M}$ of 2,4-D and $5.37 \mu\text{M}$ of NAA after 10 days. **Figure 4.1.6.2 b.** Shoot bud elongation on BAP ($4.4 \mu\text{M}$) and Kn ($1.15 \mu\text{M}$) after 30 days **Figure 4.1.6.2 c** Many shoots formed after about 45 days.

4.1.6.3 Histology of Callus (Light Microscopy)

Histological investigation revealed the formation of globular meristemoids from the nodular callus which developed into shoot bud initials. Sections of the nodular callus initially showed the presence of isodiametric cells which were smaller in size with dense cytoplasm. They got separated as globular meristemoids from the rest of the loosely arranged tissue when observed after 15 days of shifting the nodular callus to light conditions (Figure 4.1.6.3 a) and got matured after 20 days as seen in Figure 4.1.6.3 b. Maturation of globular meristemoid led

to the formation of shoot apical meristem by numerous anticlinal and periclinal divisions. It was evident by the presence of numerous layers of well organized cells in the shoot apical region as seen after 30 days (Figure 4.1.6.3 c) forming well developed shoot apical meristem after 40 days (Figure 4.1.6.3 d).

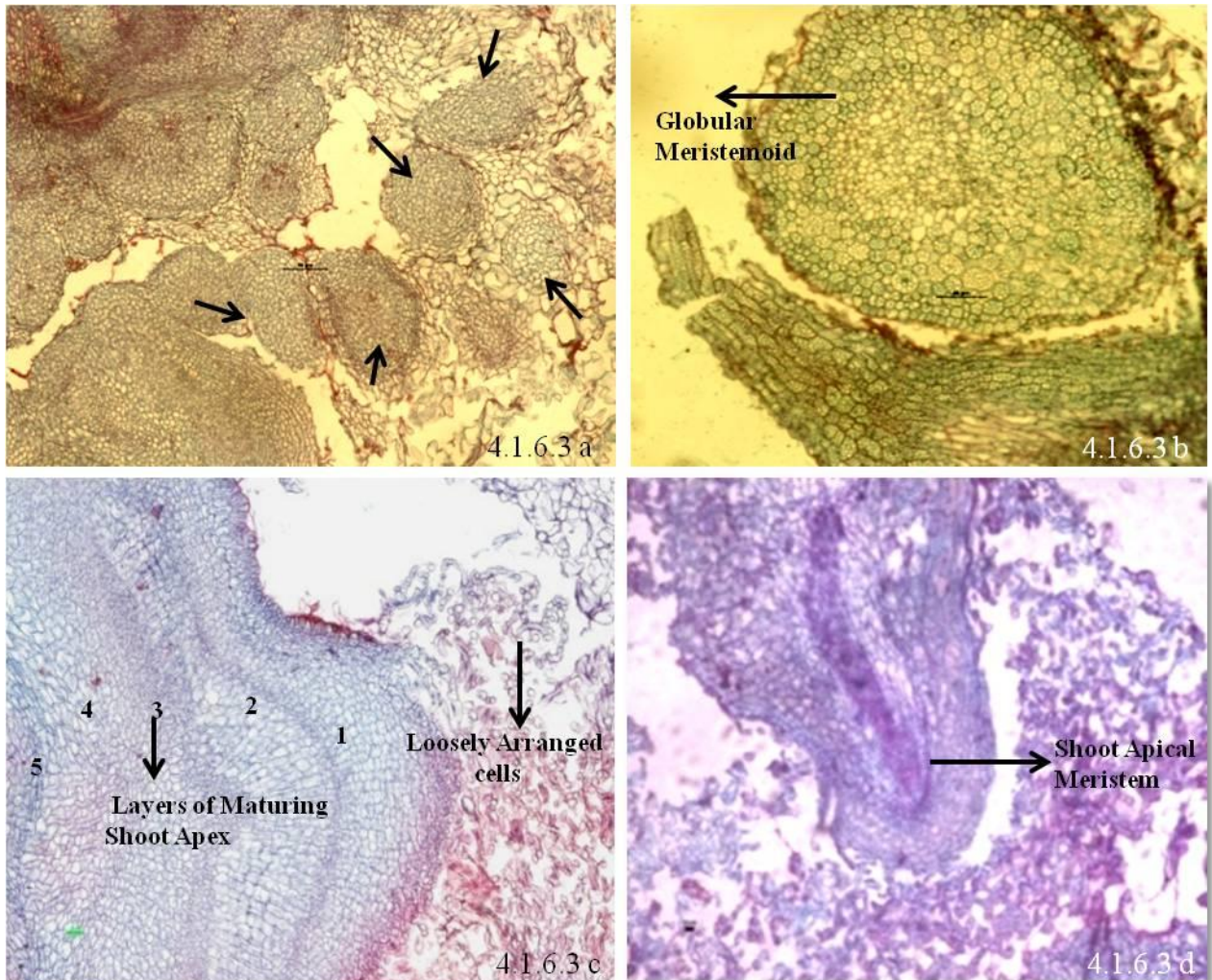


Figure 4.1.6.3 a. Numerous globular meristemoids formed 15 days after shifting the callus to light (100X). **Figure 4.1.6.3 b.** An enlarged globular meristemoid after 20 days (400 X). **Figures 4.1.6.3 c and d.** Development of shoot apex after 30 days and 40 days respectively (400X).

4.1.6.4 Histology of Callus (Scanning Electron Microscopy)

To understand more about the differentiation phenomenon, Scanning Electron Microscopy (SEM) of nodular callus at different stages of growth was carried out. Initially the cells of the nodular callus were seen as outgrowths from the surface of the callus (Figure 4.1.6.4 a) which later developed into shoot buds. We noticed the presence of shoot buds having leaf primordia as shown in Figure 4.1.6.4 b. which later developed into shoots.

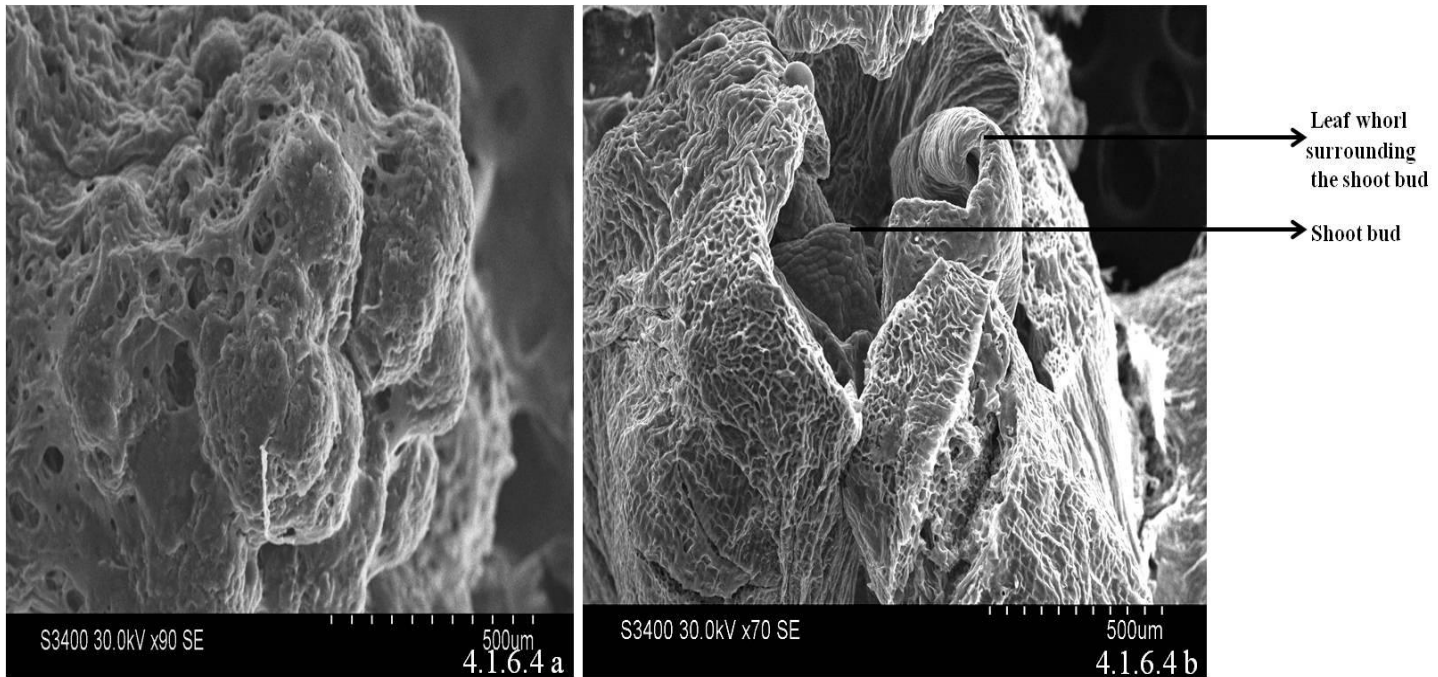


Figure 4.1.6.4 a. Scanning Electron Microscope (SEM) image showing nodular callus.

Figure 4.1.6.4 b. Shoot bud surrounded by leaf primordia seen after 40 days.

4.1.6.5 Rooting and Acclimatization

Rooting was obtained simultaneously from shoots on the shoot induction medium containing BAP (4.4 μM) in combination with Kn (1.15 μM) after 30 days without addition of any auxin. For acclimatization, plantlets were transferred to plastic covers/pots containing river bed sand and covered with plastic pots and kept in growth room initially for 2 weeks. Thereafter, they were shifted into potting mixture containing sand: soil: farmyard manure (1:1:1) and kept in greenhouse for acclimatization before transferring them to the field.

4.1.7 Seed Culture

Propagation through seeds is very important because it not only maintains genetic diversity within the population but is also simple and economical method for producing large number of plants. Use of seed in micropropagation is useful in multiplying those plants which produce seeds in uneconomical amounts or when plants do not produce viable seeds or when seed cannot be stored i.e. [recalcitrant seeds](#).

4.1.7.1 Initiation of Aseptic Cultures

For the present experimentation, seeds were procured from China and the seeds were more than 6 months old i.e. ageing seeds. Seeds are broadly ovate, rounded at base with pointed

ends, 5-7.5 mm long and dark brown in colour. They are single seeded and testa is fused with thin pericarp (Figure 4.1.7.1 a).



Figure 4.1.7.1 a. Seed showing rounded base with a pointed end.

For seed culture, the original seeds were subjected to repeated washings after removal of glumes to remove all the adhering dust particles and microbes from the surface. The explants were then cleaned with a liquid detergent followed by treatment with a fungicide bavistin (0.25%), broad spectrum antibiotics like streptomycin sulphate (0.04%) and tetracycline (0.01%) for 30 minutes. The seeds were further sterilized using two different sterilants. Different concentrations of mercuric chloride (0.04 to 0.1%) were used either alone or followed by varying concentrations (10, 15 and 20%) of sodium hypochlorite (NaOCl). Out of various combinations of these microbial disinfectants tried, 0.1% mercuric chloride (HgCl_2) for 10 minutes followed by 15% NaOCl treatment for 10 minutes gave the best results with $77.8 \pm 9.6\%$ cultures getting successfully established (Figure 4.1.7.1 b). It has also been observed that a combination of two sterilants at a moderate concentration when used for a longer duration was more effective rather than high doses used for a shorter time. After sterilization seeds were inoculated on MS medium for further experimentation.

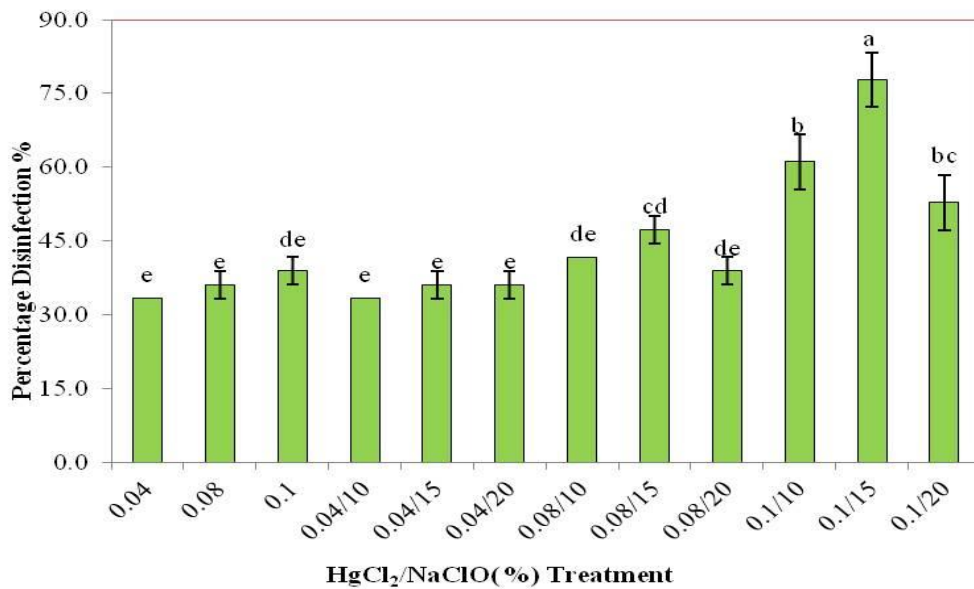


Figure 4.1.7.1 b. Different sterilization treatments for disinfection of seeds. The results were repeated thrice with 10 seeds in each replicates.

4.1.7.2 Effect of Light

Initially seeds were incubated in continuous dark for 1 week for sprouting. Protrusion of radicle > 2mm was taken as scale for germination which was observed after 7 days (Figure 4.1.7.2 a). Presence of light significantly increased the time i.e. 17.0 ± 2.0 days required for emergence of radicle. After initiation of germination, cultures were shifted to light subjecting them to a photoperiod of 16 h light/8 h dark for plantlet development and normal growth. Figure 4.1.7.2 b shows the plumule emerging out from the micropylar end which eventually grew into a shoot. Figure 4.1.7.2 c shows enlarged radicle with numerous root hairs.



Figure 4.1.7.2 a. Initiation of germination by emergence of a radicle (R) about 2mm long **Figure 4.1.7.2 b.** Micropylar end showing emergence of radicle (R) and plumule (P). **Figure 4.1.7.2 c.** Radicle showing numerous root hairs.

4.1.7.3 Effect of Gibberellic Acid (GA₃)

During normal seed germination, after water imbibition, the hormone GA₃ appears in the embryo and is translocated in the aleurone layer where it activates the metabolism to initiate sprouting. In the present experimentation, the effect of different concentrations of GA₃ on seed germination was investigated. Three replicates having 10 seeds each for every treatment of GA₃ varying between 10-70 ppm were taken and kept overnight. The results clearly indicated the role of GA₃ in enhancing seed germination and the best concentration was found to be 50 ppm showing a germination percentage of 73.3 ± 5.7 (Figure 4.1.7.3). After 2 weeks, a corresponding increase in shoot length (2.7 ± 0.7 mm) and number of sprouts (2.1 ± 0.7) was also observed.

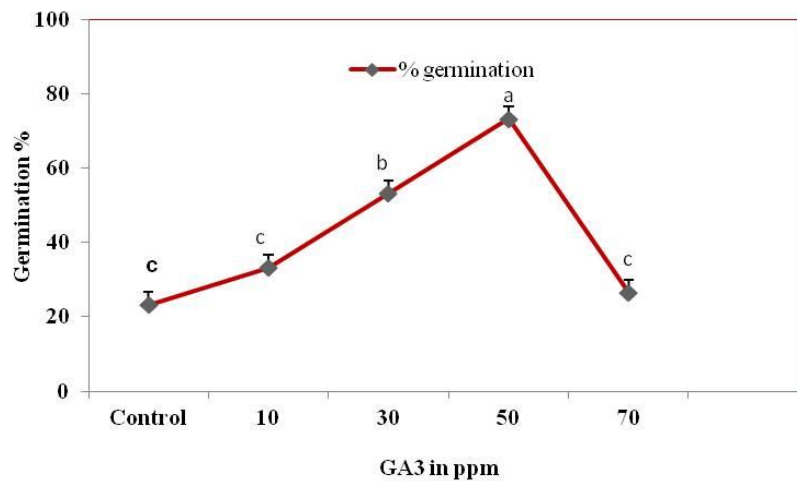


Figure 4.1.7.3 Effect of GA₃ on seed germination

4.1.7.4 Effect of Temperature

Presently, seed samples which showed delayed germination or failed to germinate under normal growth conditions, were surface sterilized and placed on wetted Whatmann No. 3 filter paper in Petridishes. Eighteen Petriplates having 6 seeds each were incubated at different temperatures 5, 10, 25, 30 and 35 °C in BOD incubators. The temperatures were selected on the basis of observation on the germination behaviour under *in-situ* and *ex-situ* conditions. Results showed absolute dependence of germination on favourable temperature. Here, 30 °C was found to be an optimum temperature at which 72.2 ± 9.6 % germination could be achieved (Figure 4.1.7.4). Low temperature of 5°C was not suitable for the ageing seeds to regain their potential for germination and growth as evident by the lowest percentage germination of 22.0 ± 9.6 .

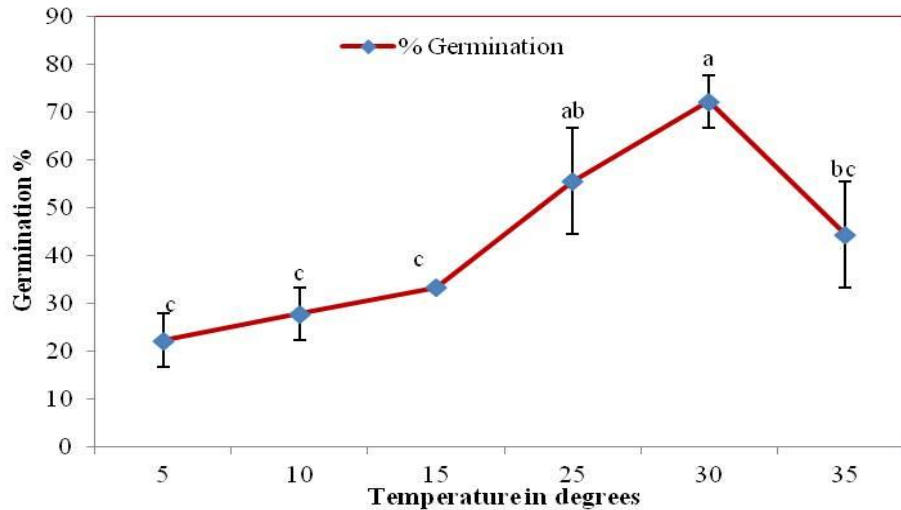


Figure 4.1.7.4. Effect of temperature on seed germination indicating 30 °C giving best response of 72.2 ± 9.6 % germination.

4.1.7.5 Effect of Growth Regulators on Multiple Shoot Proliferation from Seed Sprouts

The seeds showing sprouting on basal MS medium proliferated very slowly if kept on the same medium even for 30 days (Figure 4.1.7.5 d). Initiated cultures were therefore tested for various concentrations of BAP (2.2 to 13.2 µM) or Kn (2.3 to 13.95 µM) to study their effect on multiplication rate. Eighty five flasks representing all the treatment types including control were placed randomly on one of the shelves of culture room having uniform and controlled environmental conditions. BAP when used alone at 8.8 µM gave the best proliferation percentage of 63.0 ± 13.94 (Figure 4.1.7.5 a). Kn when used alone (2.3 to 13.9 µM) showed a poor proliferation percentage (Figure 4.1.7.5 b). However, the highest proliferation percentage of 70.0 ± 13.9 was obtained when BAP (8.8 µM) was used in synergism with 2.3 µM of Kn (Figure 4.1.7.5 c). Five replicates were taken per treatment and each experiment was repeated thrice. In all the treatments, rate of proliferation of germinated seedlings was significantly higher than the control. Proliferation of shoots on 8.8 µM of BAP and 2.3µM of Kn after 30 days and 45 days respectively is shown in Figures 4.1.7.5 e and f. Germinated plantlets growing vigorously were selected and further propagated by splitting and sub-culturing after every 20 days interval. As leaves turned yellow on medium with BAP and Kn on prolonged culturing, hence clumps were sub-cultured on basal MS medium containing 2% sucrose which turned out to be most suitable for long term proliferation of shoots. This increased their survival and retained vigour of shoots.

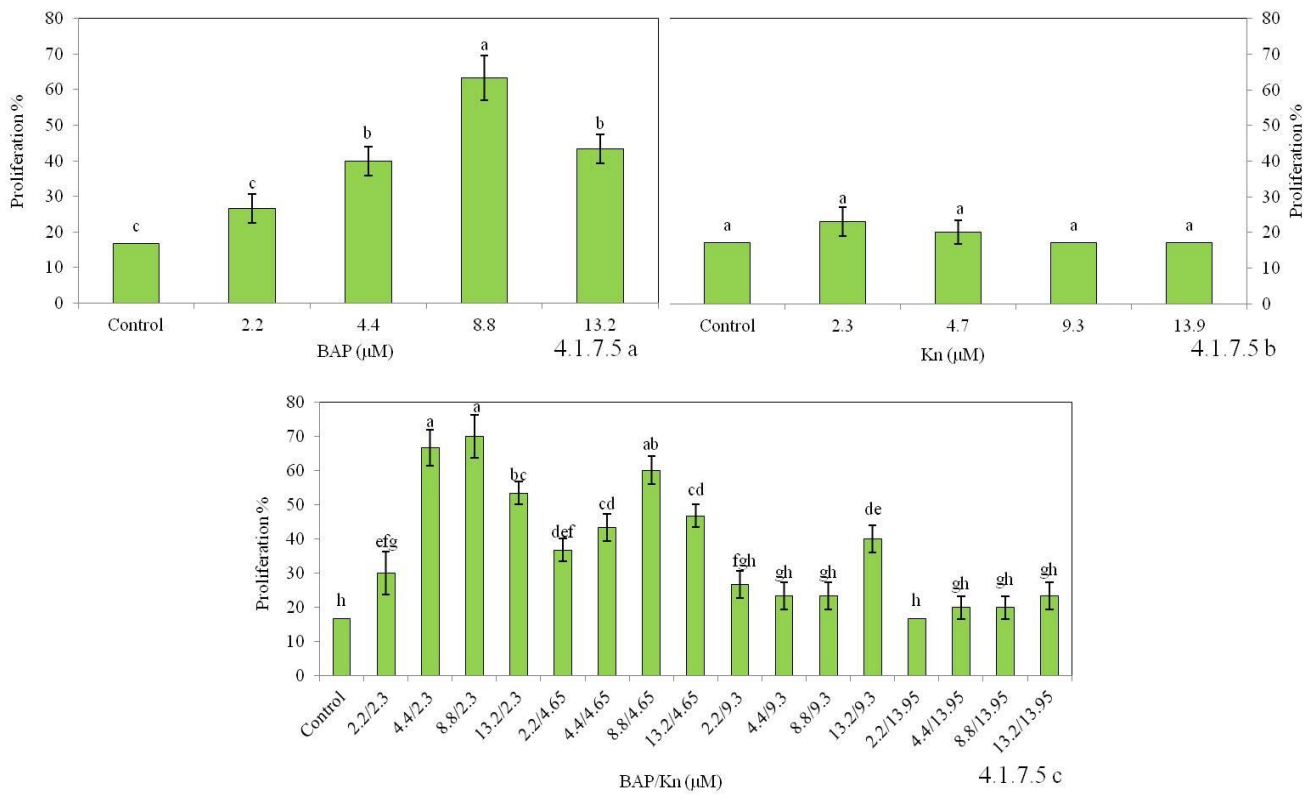


Figure 4.1.7.5 a. Percentage proliferation of shoots on BAP (2.2 to 8.8 μM). **Figure 4.1.7.5 b.** Percentage proliferation of shoots on Kn (2.3 to 13.9 μM). **Figure 4.1.7.5 c.** Percentage proliferation on combination of BAP (2.2 to 13.2 μM) along with Kn (2.3 to 13.95 μM).

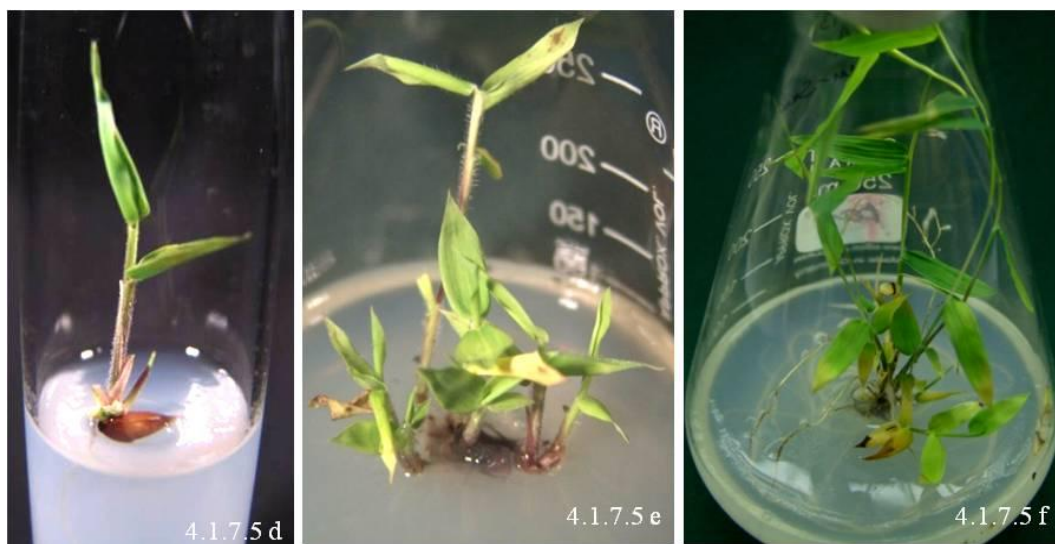


Figure 4.1.7.5 d. Sprouting of seed on Basal MS medium (BMS) after 2 weeks. **Figure 4.1.7.5 e.** Proliferation of shoots on 8.8 μM of BAP and 2.3 μM of Kn after 30 days. **Figure 4.1.7.5 f.** Increase in number of shoots on same medium after 45 days.

4.1.7.6 Rooting

It was observed that no additional auxin was required for initiation of rooting in *D. membranaceus*. Rooting was observed on 8.8 μM of BAP and 2.3 μM of Kn after 45 days (Figure 4.1.7.6 a-c). Later on plantlets were shifted to basal MS medium for healthy growth of roots.

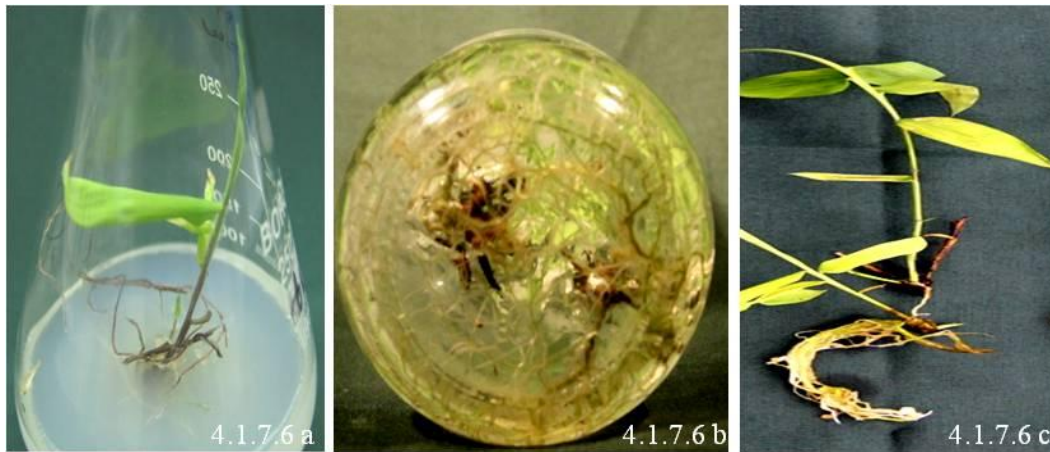


Figure 4.1.7.6 a. Root initiation on 8.8 μM of BAP and 2.3 μM of Kn after 45 days. **Figure 4.1.7.6 b** Extensive roots formed after 2 months. **Figure 4.1.7.6 c.** A complete plantlet formed.

4.1.7.7 Hardening and Acclimatization

The germinated plantlets were carefully removed from agarified medium and transferred to plastic pots containing riverbed sand and covered with perforated plastic pots/covers. They were initially kept in the growth room for 2 weeks. Afterwards, the plantlets were transferred into potting mixture containing sand: soil: farmyard manure in the ratio (1:1:1) and kept in green house (Figures. 4.1.7.7 a and b). Under green house conditions, foliage was sprayed with Hoagland solution every 7 days. The acclimatized plants had expanded leaves and showed no albinism and were successfully established in green house with 65% survival. Two months and six months old well acclimatized plants are depicted in Figures 4.1.7.7 c and d. Rainy season was found to be the best time for out planting of hardened plants in the field. Figure. 4.1.7.7 e shows hardened plants in the field which were planted in the pits (2ft \times 2ft \times 2ft) at a plant to plant and row to row distance of 6 meters. The complete protocol for micropropagation from ageing bamboo seeds of *D. membranaceus* was thus successfully established.



Figure 4.1.7.7 a. Plantlets transferred into potting mixture containing sand: soil: farmyard manure in ratio (1:1:1) for acclimatization. **Figure 4.1.7.7 b.** Hardened plant observed after 1 month. **Figure 4.1.7.7 c** Hardened plantlet after 2 months in green house.



Figure 4.1.7.7 d Hardened plants observed after 6 months.

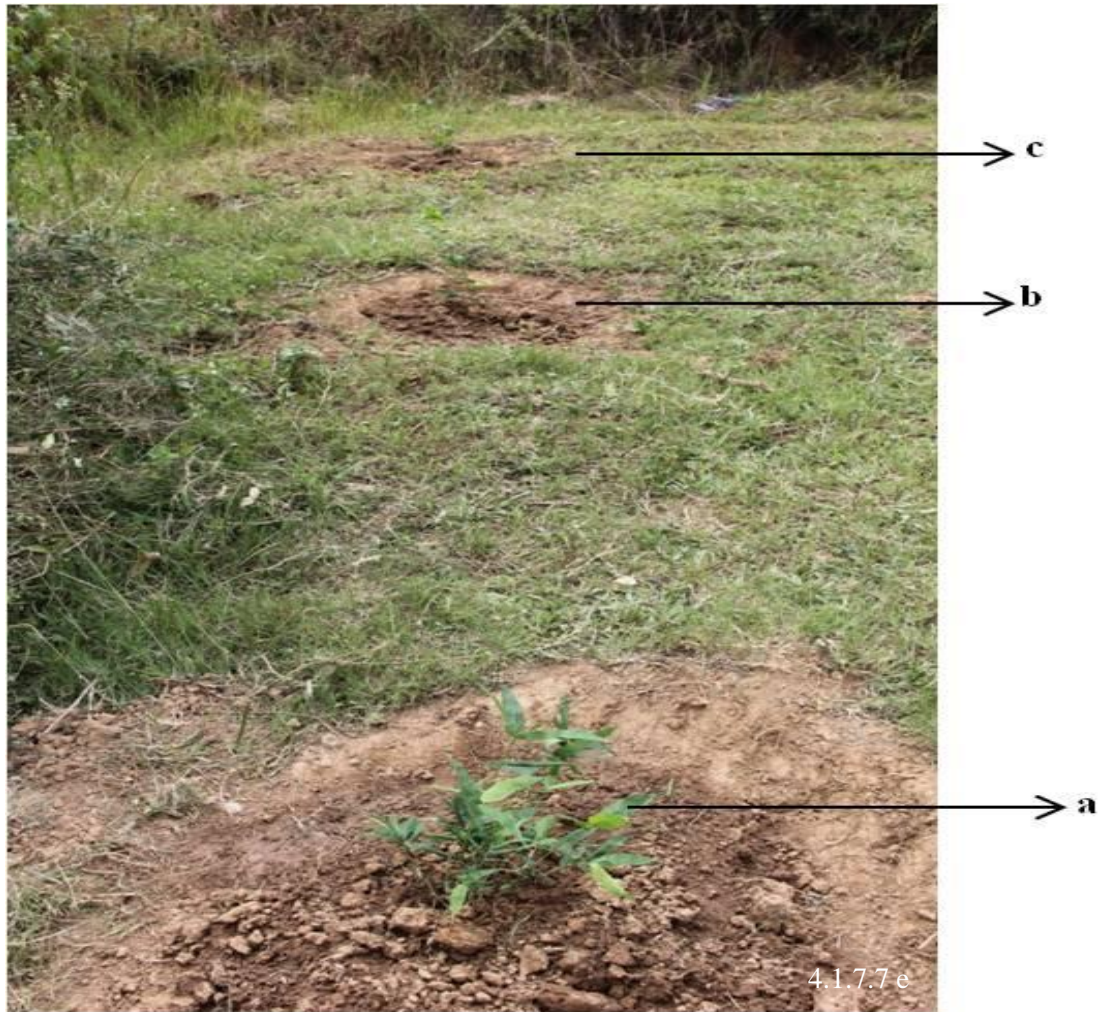


Figure 4.1.7.7 e. Micropropagated plants (a, b, c) growing in the open field conditions.

4.1.7.8 Starch Test

The seeds of bamboos have highly developed embryos and triploid endosperm comprising of aleurone layer of living cells and stored food material in the form of starch (Figure 4.1.7.8 a). In the present study, starch test was performed with three replicates having 3 seeds each to confirm the presence of starch. Appearance of deep blue colour in the areas containing starch was observed due to the presence of amylase (Figure 4.1.7.8 b). The appearance of hollow areas in the longitudinal sections of germinating seeds confirmed the consumption of starchy endosperm for growth and development of the embryo (Figure 4.1.7.8 c). Further, starch was consumed by the emergent plumule and radicle for their further growth. All those seeds not responding to any of the above treatments like GA₃, temperature and PGRs were subjected to Tetrazolium Chloride (TTC) test for checking their viability. Only seeds showing strong red stained embryos at their swollen stalk ends were considered viable and counted. The

observations recorded from the experiment by TTC test showed that after a year, 99% of the seeds became non-viable (Figure 4.1.7.8 d).

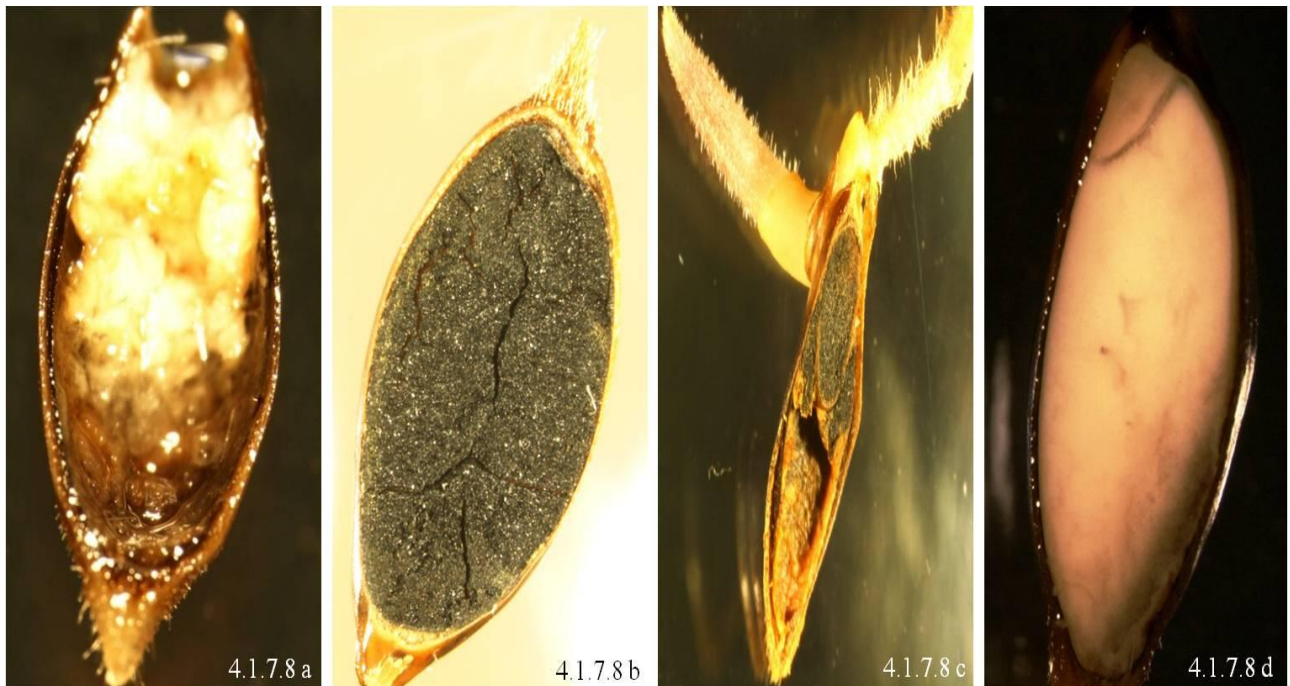


Figure 4.1.7.8 a. LS of seed showing starchy endosperm **Figure 4.1.7.8 b.** Appearance of blue black colour in areas of endosperm where starch was present. **Figure 4.1.7.8 c.** Hollow areas in endosperm depicting consumption of starch by embryo during emergence of radicle. **Figure 4.1.7.8 d.** Absence of red stain by TTC test confirming non-viable embryo.

4.1.7.9 Induction and Growth of Callus

For callus induction, the seeds were planted on MS medium augmented with various concentrations of auxins like 2, 4-D, NAA, IAA and IBA either alone or in combination with each other. Out of the different auxins tried, best callusing occurred on 4.53 μM of 2, 4-D and 5.37 μM of NAA supplemented medium where two types of calli were obtained 1) Nodular and compact, creamish white callus (2) Mucilagenous and translucent callus. The callus induction from seed occurred after 20 days of culturing (Figure 4.1.7.9 a). Mucilagenous callus was discarded because of its failure to respond for any regeneration or differentiation ability (Figure 4.1.7.9 b) and only nodular callus was retained. Nodular callus proliferated further forming a mass of callus after 40 to 45 days (Figure 4.1.7.9 c) and was capable of sustained growth on repeated subculturing. The effect of 2,4-D and NAA on callus initiation and growth is depicted in Table 4.1.7.9.

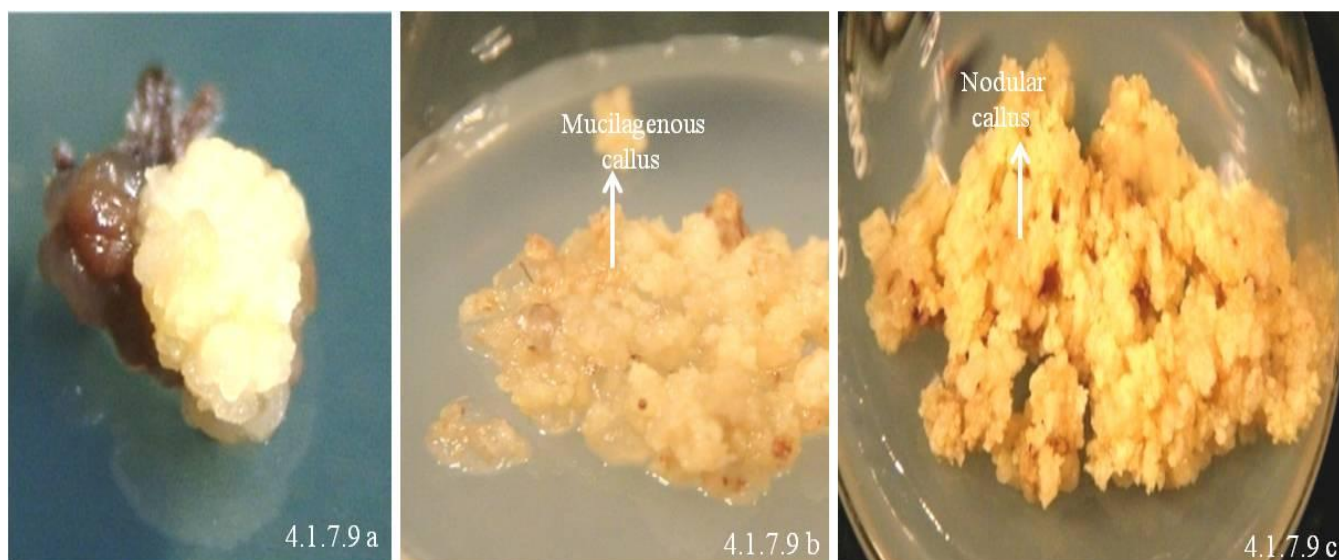


Figure 4.1.7.9 a. Callus initiation on 4.53 μM of 2, 4-D and 5.37 of μM NAA **Figure 4.1.7.9 b.** Mucilagenous callus on 4.53 μM of 2, 4-D and 5.37 μM of NAA **Figure 4.1.7.9 c.** Nodular callus showing proliferation on 4.53 μM of 2, 4-D and 5.37 μM of NAA after 40 days.

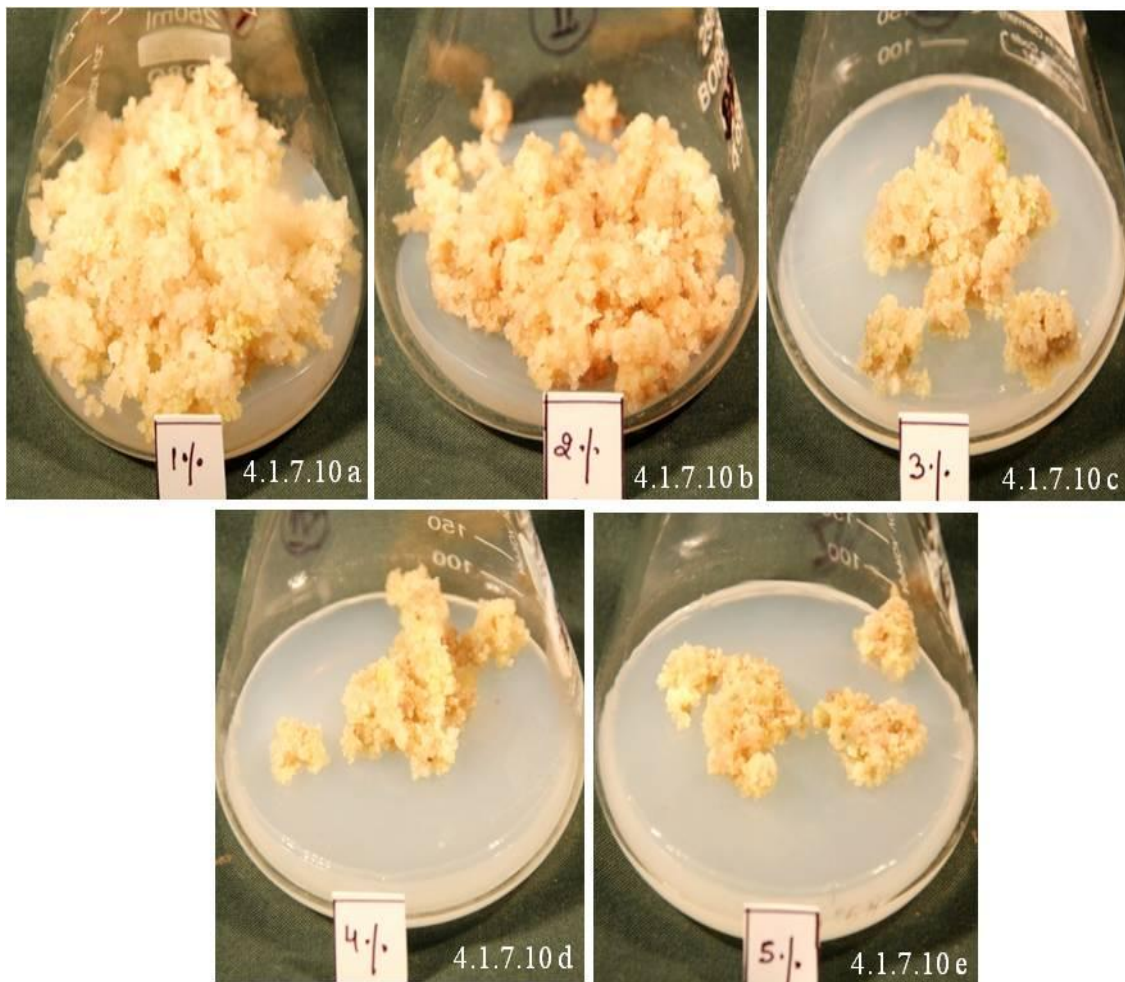
Table 4.1.7.9 Effect of Medium Composition on Callus Induction from Seeds

S. No.	Media Composition	Concentration μM	% Callus Induction	Days Required
1	MS	-	0	-
2	MS+2,4-D	0.45	30	30
3	MS + 2,4-D	2.26	50	30
4	MS + 2,4-D	4.53	60	27
5	MS + NAA	2.68	0	-
6	MS + NAA	5.37	0	-
7	MS + 2,4-D + NAA	4.53 + 5.37	90	22
8	MS + 2,4-D + NAA	9.06 + 10.74	40	25

4.1.7.10 Effect of Sucrose Concentrations on Relative Growth Rate

There was a complex web of signal interactions between sucrose concentration and the growth of the callus tissue. Experiments have demonstrated that an optimum level of sucrose was needed for callus proliferation. Growth rate studies were carried out using various concentrations of sucrose ranging from 1 to 5% as shown in [Figures 4.1.7.10 a-e](#). To measure the growth of callus, 2 months old callus with initial fresh weight of 3.0 g was inoculated on proliferating medium and incubated under light conditions of 16/8 hr cycle at $25 \pm 2^\circ \text{C}$. Best

results were obtained on 1% sucrose with a relative growth rate (RGR) of 0.46 ± 0.0 mg/g/day (Table 4.1.7.10). There was a corresponding increase in Growth Index (5.62 ± 0.50) and Cell Increase Ratio (4.62 ± 0.50). RGR kept on decreasing as the concentration of sucrose was increased to 5% keeping rest of the medium composition similar. Thus a definite correlation between sucrose concentration and RGR was established confirming the role of sucrose as the main carbon source for providing energy and increasing the biomass.



Figures 4.1.7.10 a-e Relative growth of callus on different concentrations of sucrose showing highest growth rate on 1% sucrose

Table 4.1.7.10 Effect of Sucrose Concentrations on Relative Growth Rate (RGR)

Sucrose %	RGR mg/g/day	GI	CIR	Colour of callus
1	0.46 ^a ±0.05	5.62 ^a ±0.50	4.62 ^a ±0.50	Creamish
2	0.32 ^b ±0.02	4.25 ^b ±0.24	3.25 ^b ±0.24	Creamish + light brown
3	0.24 ^c ±0.02	3.40 ^c ±0.23	2.40 ^c ±0.23	Creamish + light brown
4	0.16 ^d ±0.02	2.66 ^d ±0.23	1.66 ^d ±0.23	Creamish
5	0.11 ^e ±0.01	2.11 ^e ±0.16	1.11 ^e ±0.16	Creamish

Mean of 5 replicates. Different *uppercase* letters within the *columns* indicate significant difference among treatments (Duncan's multiple range test, $P \leq 0.05$)

4.1.7.11 Effect of Strength of MS Medium on RGR

Strength of basal MS medium was found to have effect on RGR of the callus. Hormonal concentrations of auxins (2,4-D or NAA) used were kept constant and only the strength of the medium was a variable factor. Highest RGR of callus was observed on ½ strength MS medium i.e. 0.45 ± 0.02 mg/g/day with 5.57 ± 0.23 growth rate (GI) and 2.62 ± 0.23 cell increase ratio (CIR) respectively as shown in Table 4.1.7.11. Half strength MS showed overall better response if cultures were prolonged for sub-culturing. Reducing the macronutrients of the Murashige and Skoog's formula to 1/4th of their originally recommended dose only reduced the bamboo callus growth. Colour of callus was creamish in all the three combinations (Figures 4.1.7.11 a-c).

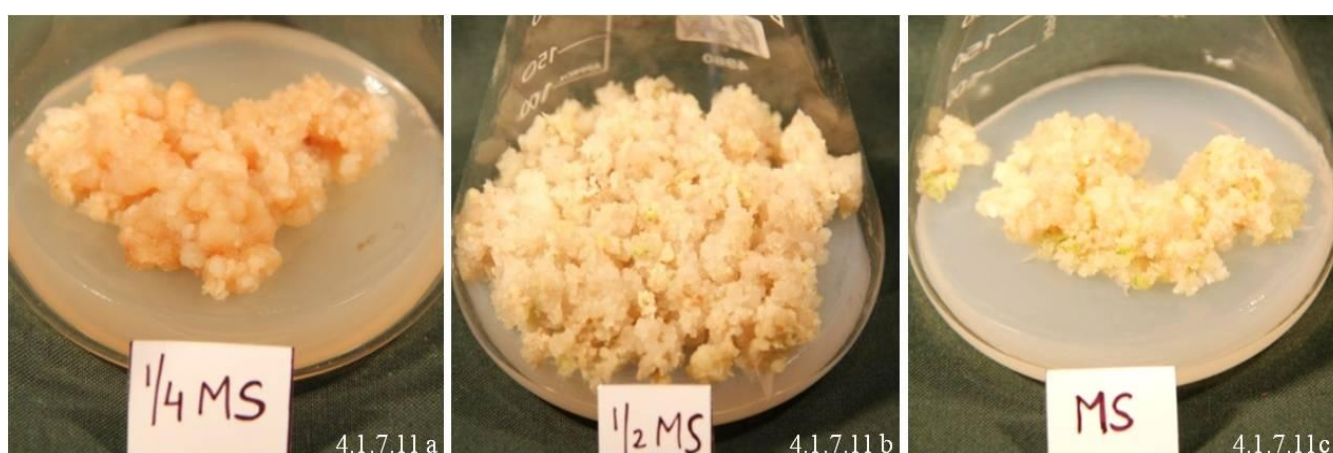


Figure 4.1.7.11 a. Callus proliferation on ¼ MS media Figure 4.1.7.11 b. Callus proliferation on ½ MS medium. Figure 4.1.7.11 c. Callus proliferation on MS full strength media.

Table 4.1.7.11 Strength of Medium and Relative growth rate

Strength of medium	RGR mg/g/day	GI	CIR	Colour of callus
1/4 th MS	0.26 ^c ± 0.01	3.62 ^c ± 0.15	2.62 ^c ± 0.15	Creamish + light brown
½ MS	0.45 ^a ± 0.02	5.57 ^a ± 0.23	4.57 ^a ± 0.23	Creamish
Full MS	0.33 ^b ± 0.02	4.33 ^b ± 0.22	3.33 ^b ± 0.22	Creamish

Mean Value of 5 replicates. Different *uppercase* letters within the *columns* indicate significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).

4.1.7.12 Effect of GA₃ on RGR

As the application of GA₃ was found to have an effect on the growth rate in many bamboo species, experiments were conducted using varying concentrations of GA₃ (1.1 to 2.8 μM) in the media (Figures 4.1.7.12 a-c) along with 4.53 μM of 2,4-D. RGR was found to be maximum i.e. 0.63 ± 0.05 mg/g/day when 2.8 μM of GA₃ was used (Table 4.1.7.12) with a GI of 7.37 ± 0.50 and a CIR of 6.37 ± 0.50 .

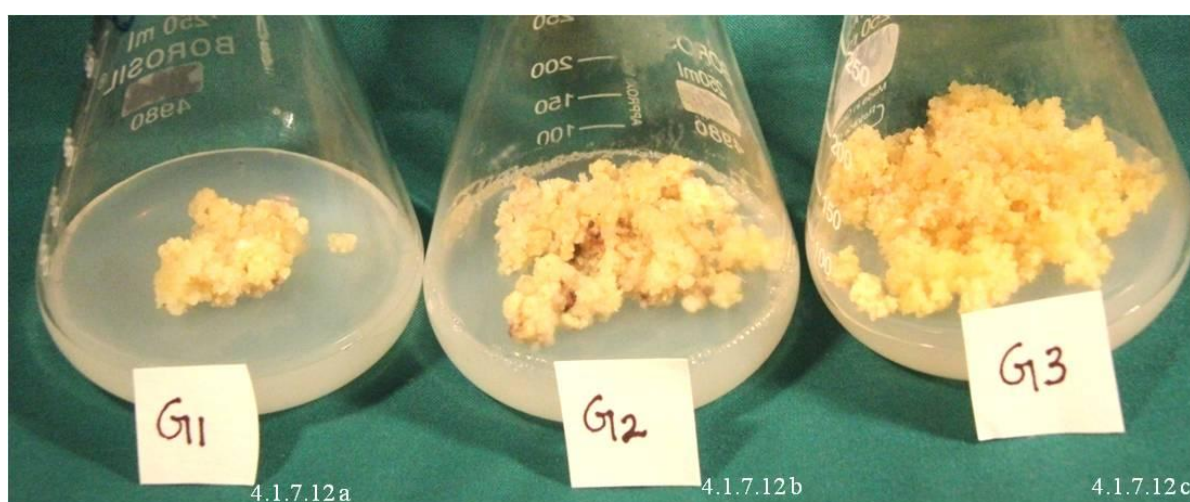


Figure 4.1.7.12 Growth of callus on a. 1.1 μM of GA₃ b. 1.7 μM of GA₃ c. 2.8 μM of GA₃.

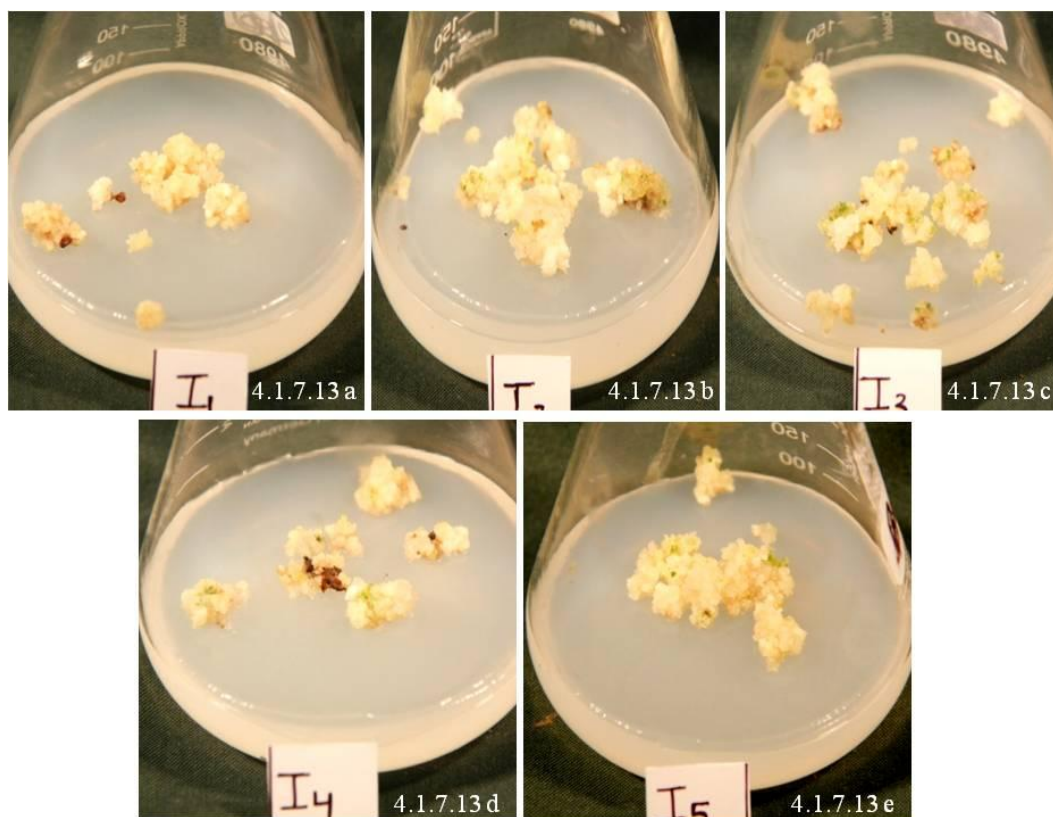
Table 4.1.7.12 GA₃ and Relative Growth Rate (RGR)

Concentration of GA ₃ in μM	RGR	GI	CIR
1.1	0.24 ^c ± 0.02	3.46 ^c ± 0.29	2.46 ^c ± 0.29
1.7	0.41 ^b ± 0.03	5.18 ^b ± 0.31	4.18 ^b ± 0.31
2.8	0.63 ^a ± 0.05	7.37 ^a ± 0.50	6.37 ^a ± 0.50

Mean Value of 5 replicates. Different *uppercase* letters within the *columns* indicate significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).

4.1.7.13 Effect of Myo-Inositol on RGR

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. Myo-Inositol is a member of B vitamin that plays a central role in growth and development. Myo-Inositol at a concentration of 300 mg/l gave the best RGR giving the highest value of 0.49 ± 0.05 mg/g/day (Figure 4.1.7.13 a-e and Table 4.1.7.13).



Figures 4.1.7.13 Growth of callus at Inositol concentration of a. 100 mg/l b. 200 mg/l c. 300 mg/l d. 400 mg/l e. 500 mg/l respectively.

Table 4.1.7.13 Effect of Myo-Inositol on RGR

Concentration mg/l	RGR mg/g/day	GI	CIR	Colour of Callus
100	0.30 ^c ± 0.03	4.01 ^c ± 0.31	3.01 ^c ± 0.31	Creamish
200	0.37 ^b ± 0.04	4.75 ^b ± 0.44	3.75 ^b ± 0.44	Creamish/green
300	0.49 ^a ± 0.05	5.97 ^a ± 0.52	4.97 ^a ± 0.52	Creamish / Green
400	0.25 ^d ± 0.03	3.5 ^d ± 0.35	2.50 ^d ± 0.35	Creamish
500	0.17 ^e ± 0.02	2.7 ^e ± 0.25	1.77 ^e ± 0.25	Creamish / Green

Mean value of 5 replicates. Different *uppercase* letters within the *columns* indicate significant differences among treatments (Duncan's multiple range test, $P \leq 0.05$).

4.1.7.14 Organogenesis from Callus

When callus was shifted to 2,4-D (4.87 μ M) in conjunction with BAP (1.13 μ M), nearly 40% callus lumps showed development of green bud like protuberance (Figure 4.1.7.14 a) but these did not grow into shoots despite continuous culturing on the same medium. Histology of callus revealed the formation of meristemoids which developed into shoot bud initials (Figures 4.1.7.14 b and c) which, however, failed to develop into shoots on any of the media tried. No stages of embryogenesis were observed.

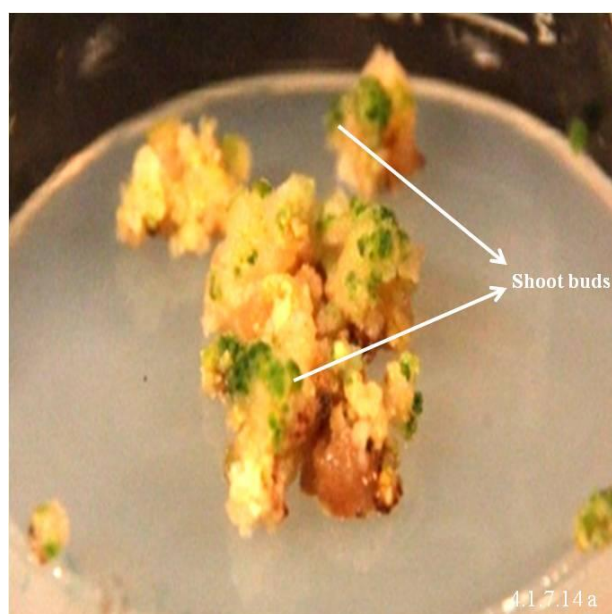


Figure 4.1.7.14 a. Formation of green protuberances on 2,4-D (4.87 μ M) + BAP (1.13 μ M) after 2 months.

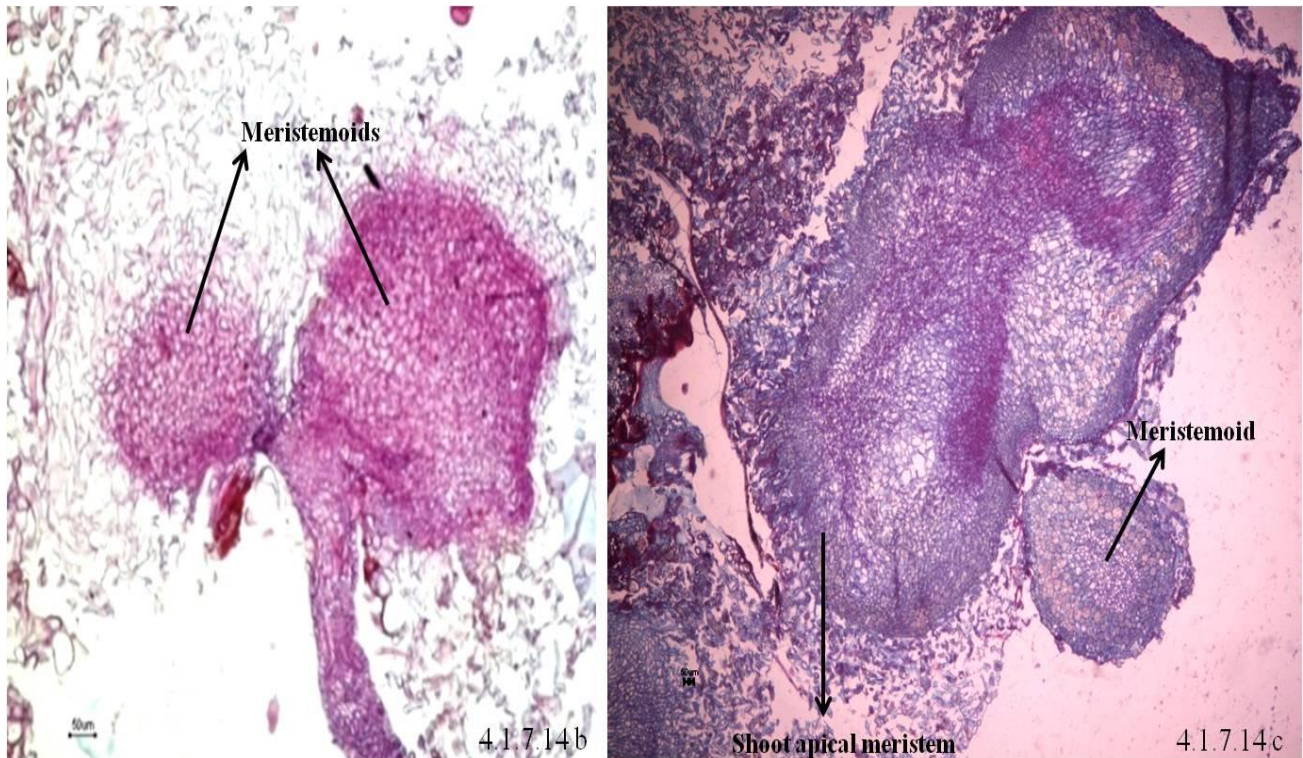


Figure 4.1.7.14 b. Section of callus showing meristemoid formation (400 X) **Figure 4.1.7.14 c.** Formation of shoot initials from the meristemoid showing the shoot apical meristem (400 X).

4.1.8 Evaluation of *In Vitro* Plants and 6 Months Old Field Established Plants

A comparative evaluation was made of 6 weeks old *in vitro* plants and 6 months old hardened plants with respect to various anatomical and biochemical parameters.

4.1.8.1 Culm Anatomy

The material was fixed, stained and observed under light microscope for studying the internal details of the culm. Studying the anatomy of culm has revealed a distinct epidermal layer and many collateral closed vascular bundles scattered in parenchymatous ground tissue. Vascular bundles were more numerous and smaller in the periphery as compared to the inner zone where they were larger sized and fewer (Figure 4.1.8.1 a). Vascular bundles consisted of xylem with one or two smaller protoxylem and two large metaxylem elements. Phloem with thin walled sieve tubes was found on the outer side of the xylem. A bundle sheath made up of sclerenchyma surrounded each vascular bundle (Figure 4.1.8.1 b).

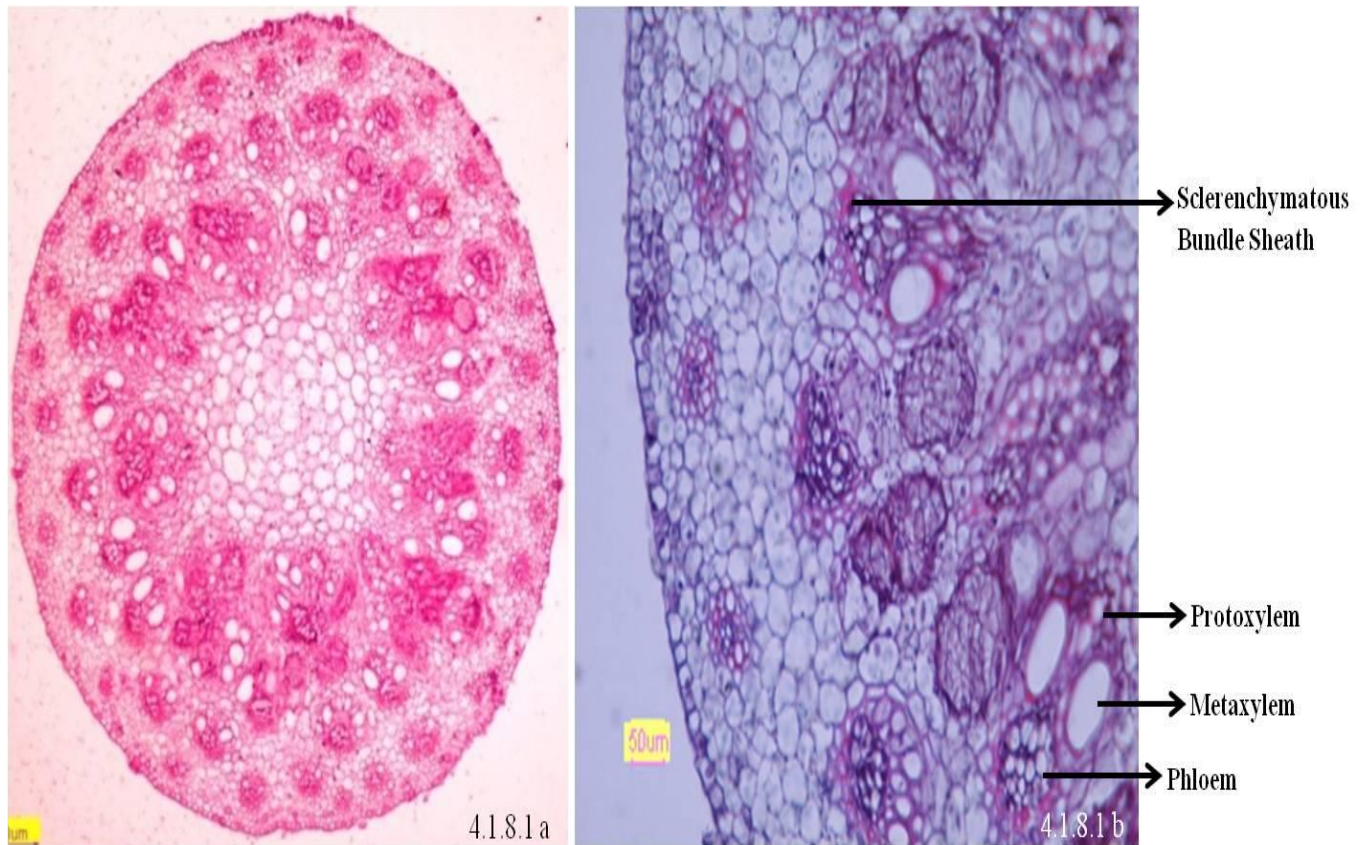


Figure 4.1.8.1 a. T.S/ Cross section culm of *D. membranaceus* showing arrangement of vascular bundles (100 X) **Figure 4.1.8.1 b.** A collateral vascular bundle showing xylem, phloem and sclerenchymatous bundle sheath (400 X).

4.1.8.2 Leaf Anatomy

Transverse section of leaf showed an upper epidermis, pallisade tissue and lower epidermis (Figure 4.1.8.2 a). Epidermal layer consisted of fan shaped bulliform cells which occupy less than half the leaf thickness. They were situated at the base of shallow furrows and made the epidermis discontinuous (Figure 4.1.8.2b). Vascular bundles were typical monocotyledonous. A group of sclerenchymatous cells interconnecting vascular bundles with upper and lower epidermis was seen in the regions of midrib (Figure 4.1.8.2 c). Fusoid cells were on both the sides of the vascular bundle. They were distinguished by their extremely thin walls and by being achlorophyllous. They were seen lying perpendicular to the vascular bundles.

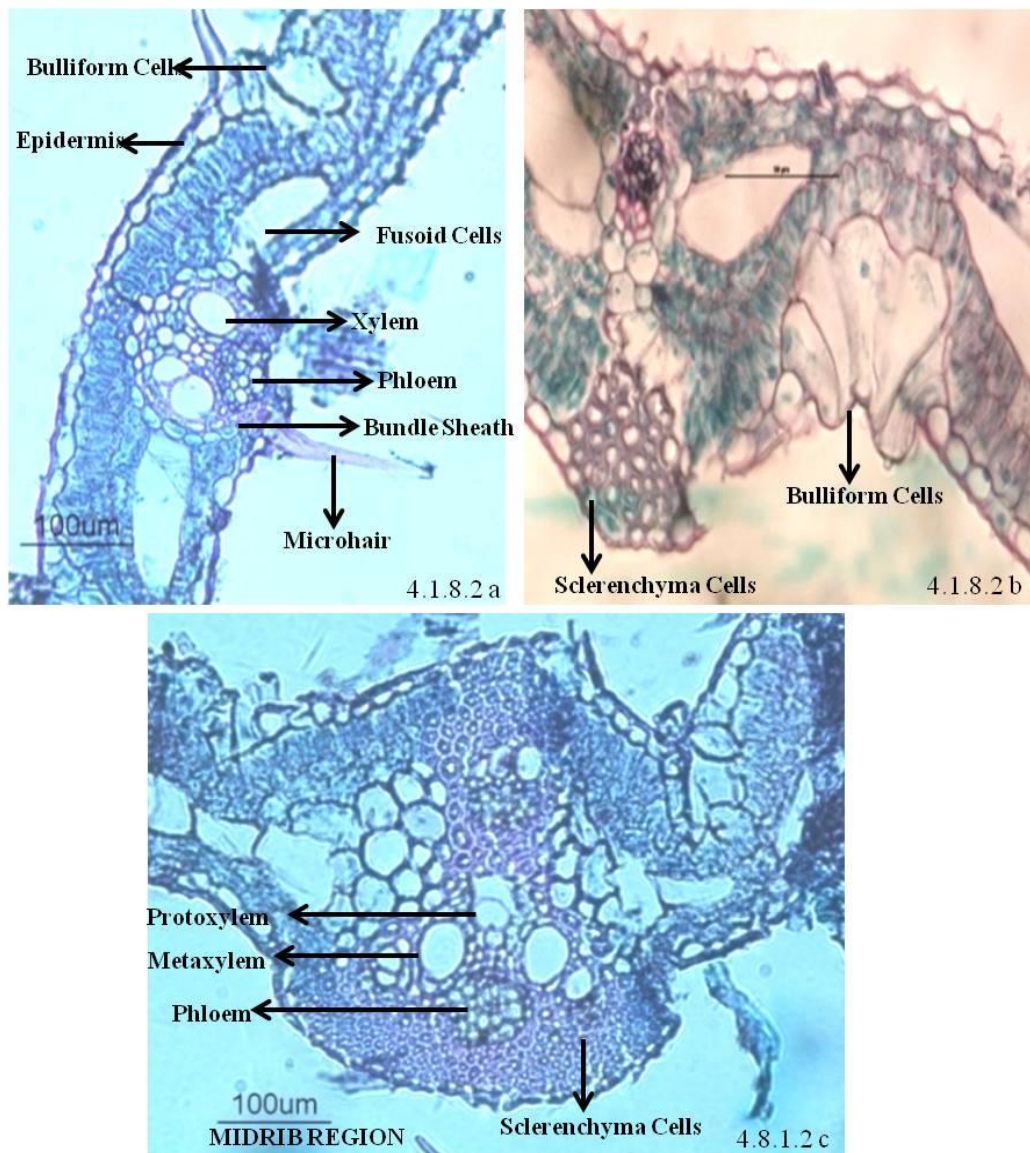


Figure 4.1.8.2 a. TS of leaf showing epidermis, palisade tissue, vascular bundle, trichome and fusoid cells (400 X) **Figure 4.1.8.2 b.** Fan shaped bulliform cells present in epidermis making it discontinuous (400X). **Figure 4.1.8.2 c** Midrib portion showing complex vasculature and sclerenchymatous cells (400 X).

4.1.8.3 Root Anatomy

Root is the least changeable of all organs of a plant and studying its anatomy holds relevance to understand a particular species. L.S. of root showed root apex and root cap region (Figure 4.1.8.3 a). Vascular bundles were arranged in the form of a ring around the pith. A layer of endodermis separated the cortex. Vascular bundles were closed and collateral (Figure 4.1.8.3 b).

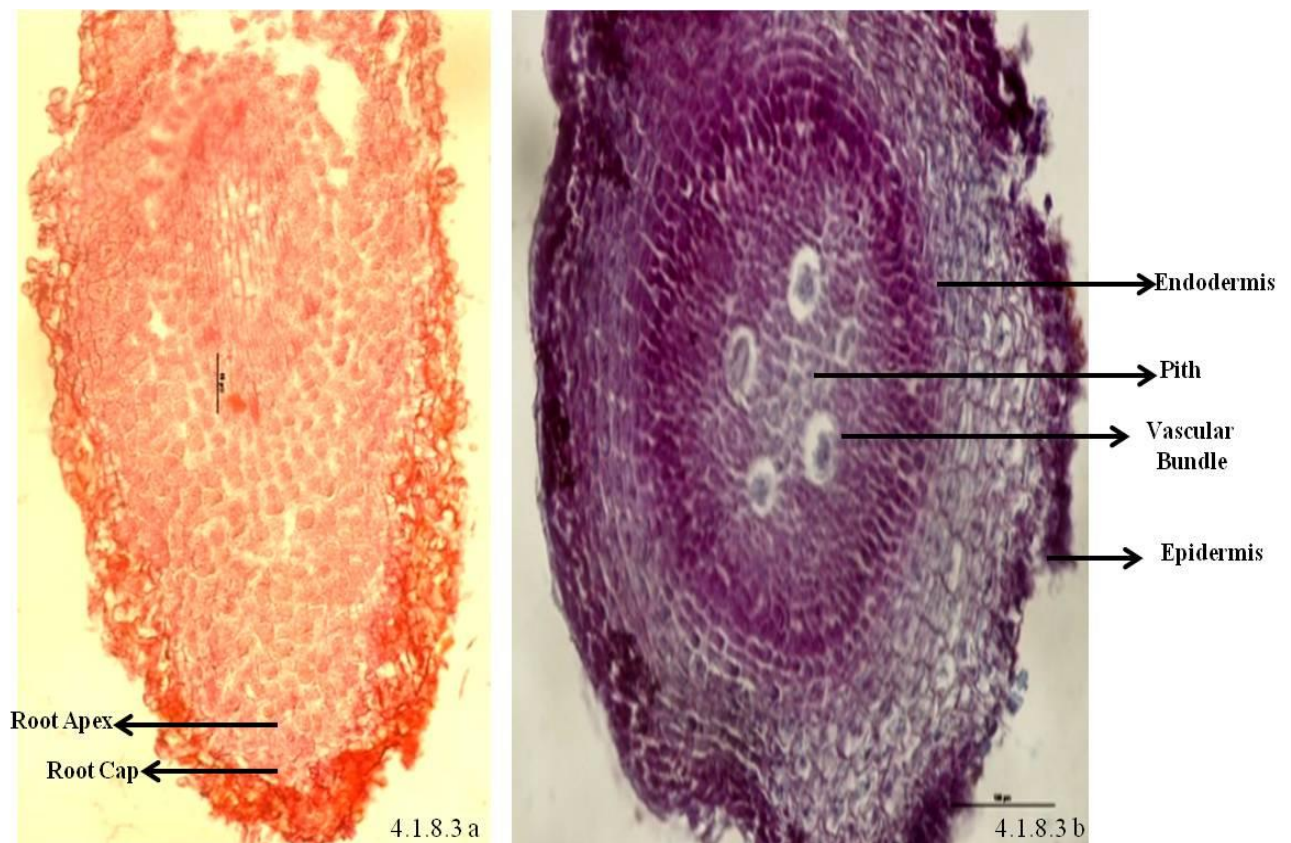
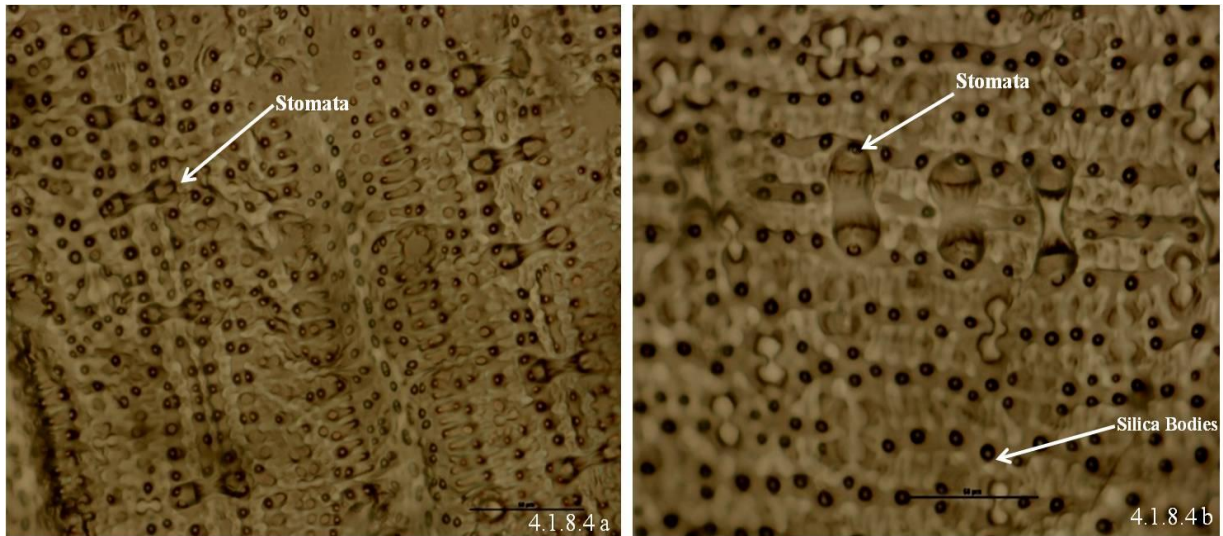


Figure 4.1.8.3 a L.S of root showing root apex and root cap region (400 X) **Figure 4.1.8.3 b** T.S of root showing internal structure (400 X).

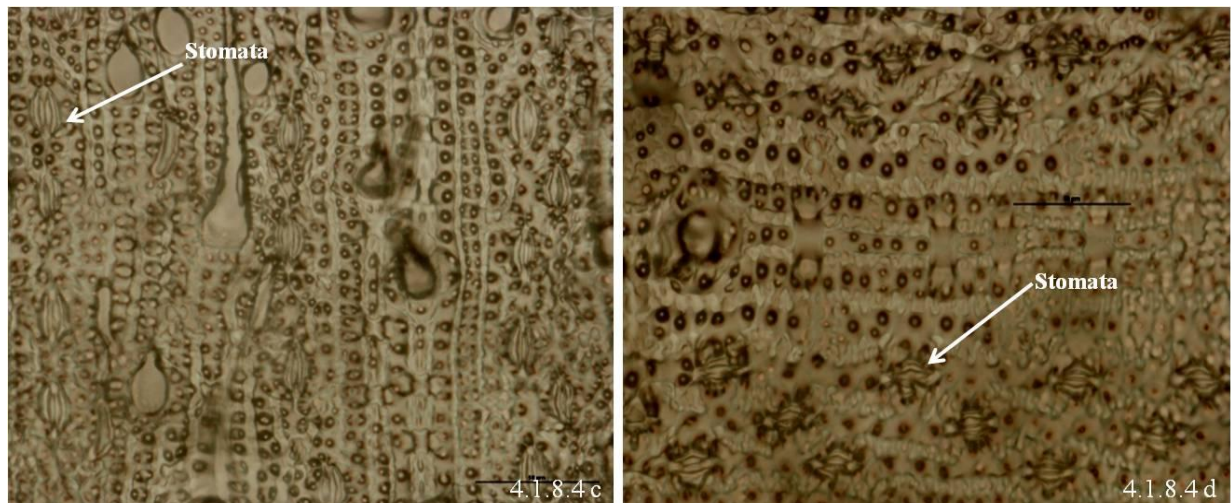
The anatomical study of culm, leaf and root of *in vitro* plants did not show any major differences in their basic structure when compared to the field grown plants except for the presence of more lignified tissue in the latter.

4.1.8.4 Stomatal Studies (Light Microscopy)

Stomatal number and stomatal index were found to be more in the *ex vitro* raised plants when compared to the *in vitro* grown plants. *In vitro* grown plant had a stomatal density of 15.33 ± 1.52 in $68 \mu\text{m}^2$ on the adaxial surface (**Figure 4.1.8.4 a**) as compared to the *ex vitro* grown leaf showing a stomatal density of 19.00 ± 1.00 (**Figure 4.1.8.4 b**). Similarly, stomata on abaxial surface of *in vitro* grown plant has a density of 25.33 ± 1.52 (**Figure 4.1.8.4 c**) as compared to 29.33 ± 1.52 (**Figure 4.1.8.4 d**) of *ex vitro* grown plant. It was also observed that more number of stomata were open on abaxial as compared to the adaxial side. Shape of stomata observed was typical dumb-bell shape. Silica bodies were invariably present.



Figures 4.1.8.4 a, b. Stomata on adaxial surface of leaves of *in vitro* and *ex vitro* grown plants respectively.



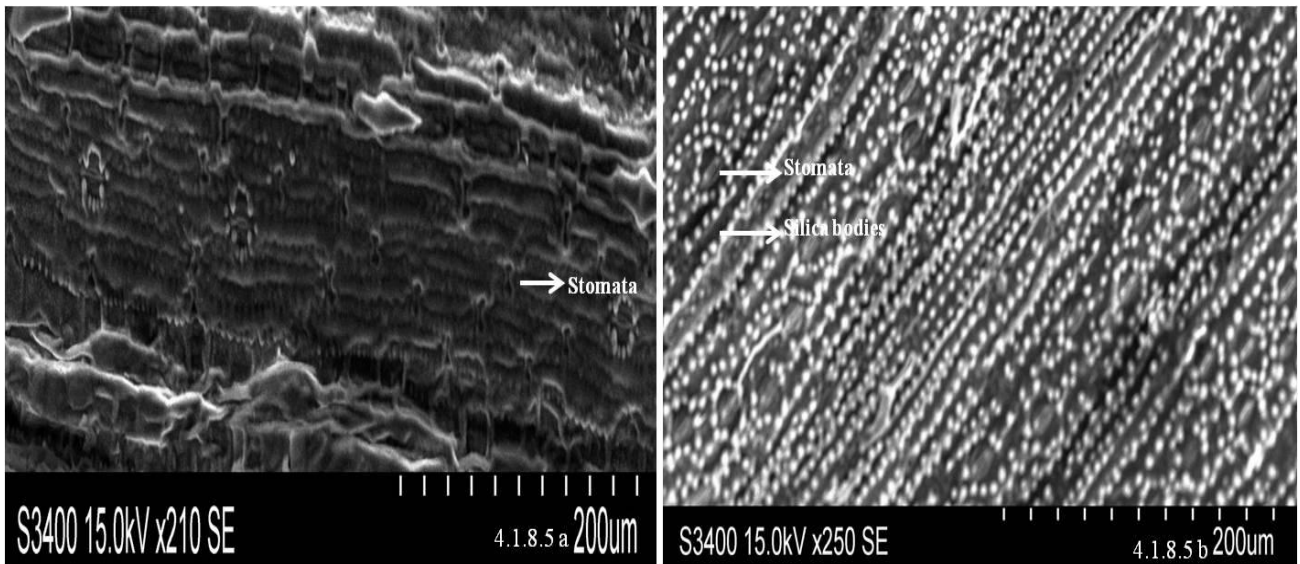
Figures 4.1.8.4 c, d. Stomatal density on abaxial surface of leaf of *in vitro* and *ex vitro* grown plant respectively

4.1.8.5 Stomatal Studies by Scanning Electron Microscopy

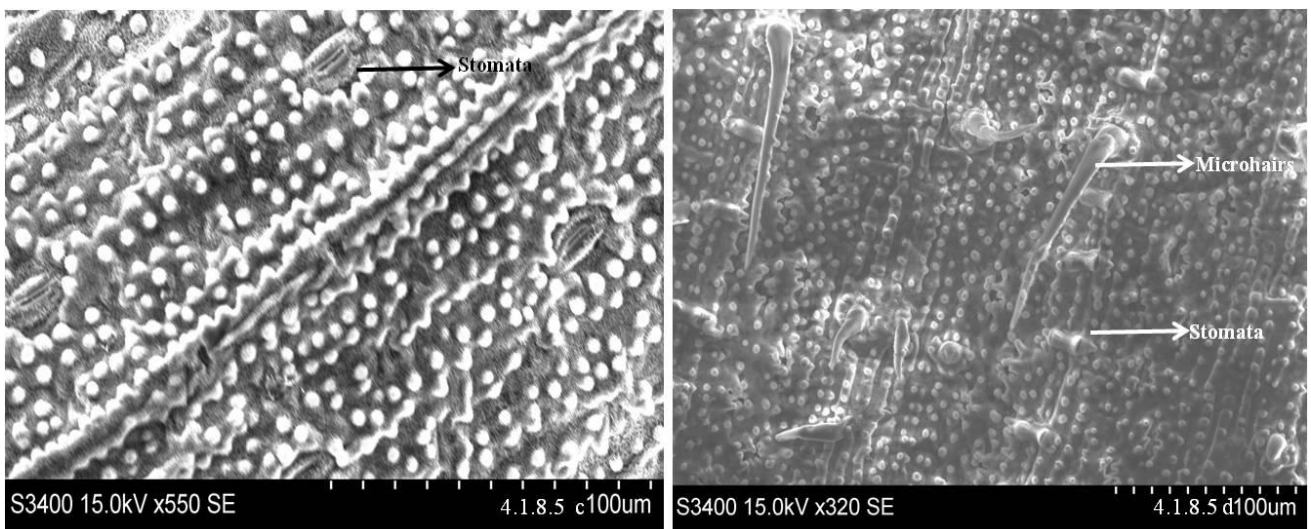
In addition to light microscopy, stomatal density was compared under the higher magnification of Scanning Electron Microscope (SEM). The electron micrograph of the adaxial surface of *in vitro* grown leaves depicted a stomatal density of 91.6 ± 7.6 in $193 \mu\text{m}^2$ (Figure 4.1.8.5 a) as compared to the stomatal density of 125 ± 15.0 in $193 \mu\text{m}^2$ (Figure 4.1.8.5 b) in *ex vitro* grown leaf. Stomata were typical dumb bell shaped and silica bodies were seen scattered here and there in the leaf mesophyll tissue. Further, it was found that abaxial surface of *in vitro* grown leaf had a stomatal density of 103 ± 12.5 in $193 \mu\text{m}^2$ (Figure 4.1.8.5 c) as compared to *ex vitro* grown leaf showing stomatal density of 131 ± 7.63 in $193 \mu\text{m}^2$. Hence, the total number of stomata per leaf were found to be more in *ex vitro* plants which is due to

more leaf area growth after transfer to *ex vitro* conditions. More number of stomata were open in the *in vitro* grown plants as compared to hardened plants. Microhairs like protective structures were seen more conspicuously in the acclimatized plants mainly on the abaxial surface in costal and intercostal regions of the leaves (Figure 4.1.8.5 d).

Scanning Electron Microscopy (SEM) Studies in *Dendrocalamus membranaceus*



Figures 4.1.8.5 a, b Scanning electron microscopy (SEM) images showing stomata on the adaxial surface of *in vitro* grown leaf and *ex vitro* grown leaf respectively.



Figures 4.1.8.5 c, d Scanning electron microscopy (SEM) images showing stomata on the abaxial surface of *in vitro* grown leaf and *ex vitro* grown leaf respectively. Microhairs were seen on the abaxial surface of leaf.

4.1.8.6 Leaf Area Index (LAI)

Leaf Area Index was used to predict photosynthetic primary production, evapotranspiration and as a tool for plant growth. LAI values for the *ex vitro* hardened plants was found to be more i.e. 5.20 ± 0.65 as compared to *in vitro* grown plants having a value of 1.35 ± 0.49 as shown in [Table 4.1.8.6](#).

4.1.8.7 Relative Water Content (RWC) and Water Saturation Deficit (WSD)

RWC and WSD % depicted plant water status in terms of cellular hydration. RWC percentage was found to be higher in the *in vitro* grown plants with value of 68.8 ± 4.09 % ([Table 4.1.8.6](#)). Low RWC (56.6 ± 3.2) % in the *in vivo* conditions can be explained from the fact that there occurs a shift in relative humidity from a higher 85% in the culture laboratory to lower ~60% in green house. This directly affected the leaf conductance, gas exchange, net assimilation rates and photosynthesis of the *ex vitro* grown plants. However, water saturation deficit (WSD) shows an inverse relationship with RWC. *In vitro* grown plants showed a value of 31.2 ± 4.09 % as compared to 43.4 ± 3.2 % in the *ex vitro* grown plants.

4.1.8.8 Electrolyte Leakage

Electrolyte leakage is a measure of poor cell integrity. The exposure of plants to *ex vitro* conditions (stressed environment) resulted in a significant increase of 76.0 ± 3.9 % of the electrolyte leakage ([Table 4.1.8.6](#)) as compared to *in vitro* grown plant. Through these preliminary experiments, it was made clear that plants tolerate various environmental stresses by loss of solutes.

4.1.8.9 Proline Estimation

Dendrocalamus membranaceus being an edible bamboo, proline is an important amino acid in nutrition. Proline accumulation in *ex vitro* plants was found to be higher (2.68 ± 0.36) $\mu\text{M/g}$ as compared to the *in vitro* (2.06 ± 0.04) $\mu\text{M/g}$ plants ([Table 4.1.8.6](#)). It was due to the response of plants to water stress and high salinity in the natural environment. Production of more proline as a compatible osmolyte helped maintain cellular homeostasis by providing the reducing equivalents for the repair of stress-induced damage, hence maintaining proper functioning and growth of the plant.

4.1.8.10 Lignin Content

Lignin content is an important parameter in wood chemistry and an essential criterion for raw material quality in pulpwood production. In the present investigation, lignin content was found to be slightly higher (29.50 ± 1.50 mg/l) in the hardened plants as compared to the *in vitro* grown plants showing a value of 25.02 ± 0.65 mg/l ([Table 4.1.8.6](#)). Our results depicted that from functional point of view, *ex vitro* plants were more developed as lignin imparts

strength to cell wall, facilitates water transport and acts as a major line of defense against pathogens, insects and herbivores.

4.1.8.11 Starch Content

Starch content also varied in the *in vitro* grown and *ex vitro* acclimatized plants. *In vitro* grown plants showed a starch content of 3.90 ± 0.14 mg g⁻¹ fr.wt. which was more than that of acclimatized plants with value of 3.65 ± 0.15 mg g⁻¹ fr.wt. as shown in [Table 4.1.8.6](#). Low levels of starch in the *ex vitro* grown plants could be attributed to the fact that the new shoots consumed the available nutrients (starch) for their growth and development.

4.1.8.12 Total Soluble Sugars (TSS)

Total soluble sugar (TSS) content is not only the main photosynthate in higher plants, but also the main form of carbohydrate for metabolism and temporary storage. Changes in sugar content were observed during *in vitro* culture with values of 2.22 ± 0.13 mg g⁻¹ fr.wt. and *ex vitro* transferred plants showed a low level of 1.60 ± 0.32 mg g⁻¹ fr.wt. ([Table 4.1.8.6](#)). Lower levels of soluble sugars in the *ex vitro* grown plants was due to more utilization of sugars for growth and differentiation processes by the growing plant. The increase in biomass of the plant under *ex vitro* environment accounted for a decreased level of TSS under *ex vitro* conditions.

4.1.8.13 Chlorophyll Content

Maximum value for total chlorophyll and carotenoids content using ultrasonics was found to be 17.92 ± 1.33 mg g⁻¹ fr.wt. in the *ex vitro* conditions as compared to a value of 9.58 ± 0.85 mg/g fr wt in the *in vitro* plants ([Table 4.1.8.13](#)). Among the various solvents used, extraction efficiency was found to be maximum in Acetone (17.92 ± 1.33 mg g⁻¹ fr.wt.) followed by DMSO (12.98 ± 2.99 mg g⁻¹ fr.wt.) and DMF (11.25 ± 3.46 mg g⁻¹ fr.wt.) due to different stabilities of these solvents. These results revealed that the extraction efficiency of chlorophyll is related to solvent types, extraction method and properties of bamboo culm meal.

Table 4.1.8.6 Effect of *In Vitro* and *Ex Vitro* Environment on Physiological Parameters in *D. membranaceus*

Physiological Parameters	<i>In vitro</i>	<i>Ex vitro</i>	Remarks
Relative Water Content%	68.8 ^a ± 4.09	56.6 ^b ± 3.22	Plant water status in terms of cellular hydration
Water Saturation Deficit	31.2 ^a ± 4.09	43.4 ^b ± 3.22	Plant water balance
Electrolyte Leakage %	68.0 ^b ± 2.97	76.0 ^a ± 3.90	Cell membrane stability
Leaf Area Index	1.35 ^b ± 0.49	5.20 ^a ± 0.65	More growth and productivity of <i>ex vitro</i> grown plants
Proline µM/g	2.06 ^b ± 0.04	2.68 ^a ± 0.36	Role in cellular homeostasis including redox balance and energy status
Lignin mg/l	25.02 ^b ± 0.65	29.50 ^a ± 1.50	Source of fiber in food and essential raw material quality in pulpwood production
Starch Content mg g ⁻¹ fr.wt.	3.90 ^a ± 0.14	3.65 ^b ± 0.15	Important criterion for edibility of shoots
TSS mg g ⁻¹ fr.wt.	2.22 ^a ± 0.13	1.60 ^b ± 0.32	Play important role in carbohydrate metabolism and for edibility of shoots

Table 4.1.8.13 Chlorophyll Content Estimation Using Different Solvents by Ultrasonics

Solvents	<i>In vitro</i> (mg/g fr.wt.)	<i>Ex vitro</i> (mg/g fr. wt.)	Remarks
Acetone	9.58 ^a ± 0.83	17.92 ^a ± 1.33	More stable and less abrasive solvent
DMSO	7.15 ^b ± 0.28	12.98 ^b ± 2.99	Less toxic than DMF
DMF	6.00 ^c ± 0.54	11.25 ^b ± 3.46	More viscous and less stable

Values are mean ± SD. ^{a,b,c} are mean separation within columns by Duncan's multiple range test, P ≤ 0.05.

4.2 Micropropagation studies in *Bambusa balcooa*

4.2.1 Initiation of Aseptic Cultures

4.2.1.1 Choice of Explants

For initiating aseptic cultures, various explants like nodal explants, apical buds and leaf bases were taken from precocious branches of 4 year old field grown healthy, disease free plant maintained at the experimental farm at CSIR-IHBT-Palampur. Explants were washed under running tap water for 25-30 minutes and further cleaned with the teepol (Tween 20) with the help of sable hair brush. This was followed by treatment with antifungal agent bavistin (0.1 to 1%) and antibacterial agents like streptomycin sulphate (0.02 to 1%) and tetracycline (0.01 to 0.1%) for another 25-30 minutes. Bavistin at 0.25%, streptomycin sulphate at 0.02 % and tetracycline at 0.02% were found to be the most effective treatments. In the laminar air flow, the explants were further washed with 70% alcohol for 1 minute followed by treatment with 15 % NaOCl for 8 minutes and final disinfection with HgCl₂ (0.1% w/v) was done for 8 minutes in case of nodal explants, 6 minutes for apical buds and 5 minutes for leaf bases.

Apical buds and leaf bases did not respond on any of the media tried and turned necrotic after sometime. However, the nodal segments taken from tertiary branches proved to be the most favourable for initiating aseptic cultures as they responded favourably to different media combinations.

4.2.2 Nodal Explants

4.2.2.1 Effect of Explant Size

Size of explant had direct effect on culture initiation and bud sprouting. The larger explants (25 mm) gave the best response for sprouting which started after 14.5 ± 1.9 days of culture whereas explants of the size 5 mm took maximum time i.e. 25.9 ± 2.3 days for sprouting (Figure 4.2.2.1). Moreover, the larger explants showed a much better response to survive all the treatments of the chemicals for sterilization whereas as smaller explants did show a tissue damage at the ends.

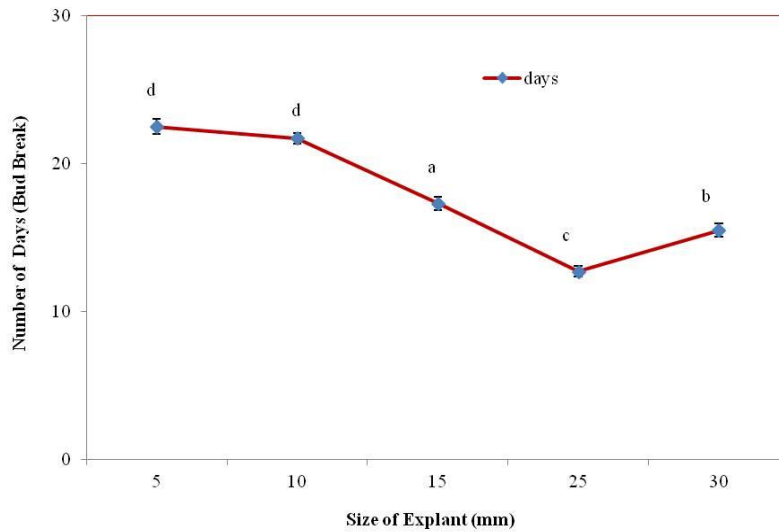


Figure 4.2.2.1. Effect of explant size on bud break.

4.2.2.2 Effect of Plant Growth Regulators on Shoot Multiplication

For axillary bud break, nodal explants were inoculated on basal MS medium containing 1% sucrose where sprouting occurred after 10-12 days (Figure 4.2.2.2 a) but only 3-4 shoots were formed which did not multiply further. For more shoot proliferation, the sprouted buds were transferred onto MS medium containing different concentrations and combinations of cytokinins like BAP (1.1 to 17.6 μM) and Kn (1.16 to 9.3 μM) either alone or in conjunction with lower concentrations (0.53 to 2.68 μM) of auxin NAA (Figure 4.2.2.2 b). Axillary shoot proliferation occurred on MS medium supplemented with 4.4 μM of BAP forming 16.8 shoots after 8 weeks. A significant increase in the shoot number was observed when BAP was used in conjunction with NAA. MS medium supplemented with BAP (4.4 μM) and NAA (0.53 μM) produced maximum number of 19.8 ± 1.4 shoots having a shoot length of 3.44 ± 0.23 cm and 19.5 ± 1.26 leaves after 60 days of culture (Figure 4.2.2.2 c). Higher doses of NAA (2.68 μM) induced thin leaf like shoots, many of these did not develop further. The effect of different concentrations of BAP and NAA on shoot proliferation is depicted in Table 4.2.2.2. The shoots thus formed were further subdivided into smaller clumps each having 3 shoots and sub cultured onto fresh multiplication medium for shoot proliferation and to avoid necrosis of shoots.

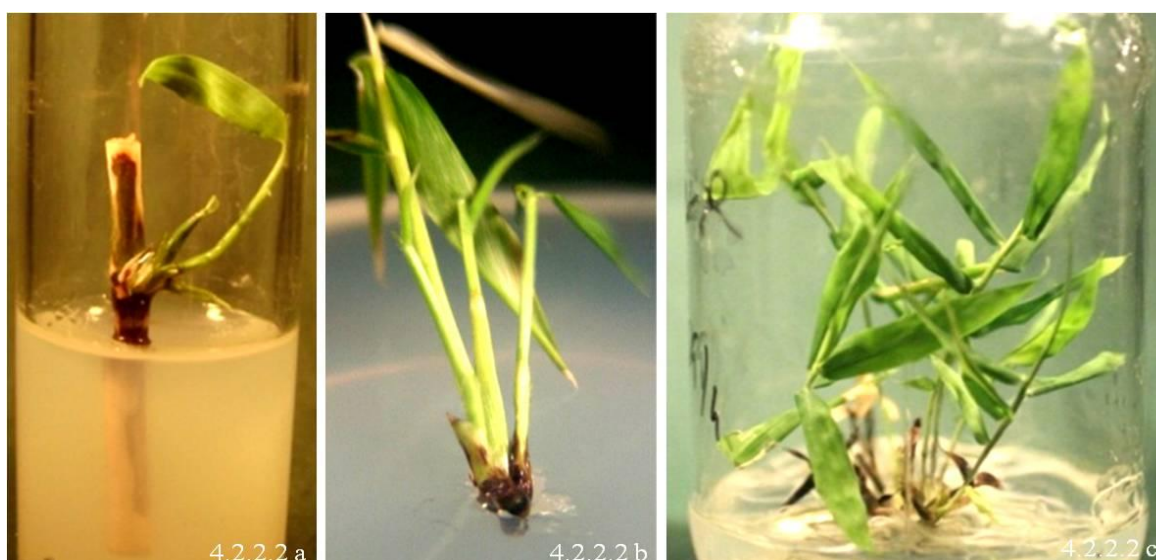


Figure 4.2.2.2 a. A sprouted bud on basal MS medium after 15 days **Figure 4.2.2.2 b.** A clump of 3-4 shoots sub-cultured onto shoot proliferation medium **Figure 4.2.2.1 c.** A cluster of axillary shoots formed on BAP (4.4 μM) and NAA (0.53 μM) after 60 days.

Table 4.2.2.2 Effects of different concentrations of PGR's on shoot proliferation

BAP+ NAA (μM)	No of Shoots	Shoot Height (cm)	Total no of Leaves
0.0+ 5.3	6.20 ^h \pm 1.39	1.23 ^g \pm 0.18	6.9 ⁱ \pm 1.59
0.0+ 1.14	4.20 ⁱ \pm 0.91	1.12 ^{gh} \pm 0.09	5.5 ^j \pm 1.17
0.0+ 2.68	3.00 ^j \pm 0.66	0.94 ^h \pm 0.14	3.9 ^k \pm 1.59
2.2+ 0.0	8.80 ^g \pm 1.39	1.54 ^f \pm 0.16	11.5 ^{fg} \pm 1.50
2.2+ 0.53	14.2 ^c \pm 1.39	1.92 ^{de} \pm 0.10	13.6 ^{de} \pm 1.71
2.2+ 1.14	10.9 ^{ef} \pm 1.10	1.75 ^{ef} \pm 0.16	13.4 ^{de} \pm 1.77
2.2+ 2.68	10.0 ^f \pm 1.55	1.60 ^f \pm 0.19	10.2 ^{gh} \pm 1.39
4.4+ 0.0	16.8 ^b \pm 1.13	2.84 ^b \pm 0.42	16.5 ^b \pm 1.50
4.4+ 0.53	19.8 ^a \pm 1.47	3.44 ^a \pm 0.23	19.5 ^a \pm 1.26
4.4+ 1.14	15.1 ^c \pm 1.91	2.40 ^c \pm 0.22	14.6 ^{cd} \pm 1.64
4.4+ 2.68	14.8 ^c \pm 1.61	2.03 ^d \pm 0.17	14.4 ^d \pm 0.84
8.8+ 0.0	14.9 ^c \pm 2.07	2.11 ^d \pm 0.27	15.9 ^{bc} \pm 1.66
8.8+ 0.53	12.7 ^d \pm 1.25	1.66 ^g \pm 0.21	14.2 ^d \pm 2.74
8.8+ 1.14	11.7 ^{de} \pm 1.25	1.62 ^g \pm 0.18	12.6 ^{ef} \pm 1.26
8.8+ 2.68	8.70 ^g \pm 1.49	1.55 ^g \pm 0.27	11.9 ^f \pm 1.52

Values are mean \pm SD. Uppercase letters ^{a,b,c,d,e,f} are mean separation within columns by Duncan's multiple range test, $P \leq 0.05$

4.2.2.3 Effect of Sucrose on Shoot Multiplication

Sucrose was the most commonly used organic compound in the nutrient medium serving as energy source. To study the effect of different concentrations of sucrose on shoot multiplication, three shoots/clump were cultured on MS medium containing BAP (4.4 μM) and NAA (0.53 μM) along with different concentrations of sucrose (0.5 to 3%). Out of different concentration of sucrose tried, 1% sucrose was found to be the best where maximum shoot proliferation occurred. We recorded on an average of 19.8 ± 1.4 shoots after 7 weeks of culture (Figure 4.2.2.3). Replacement of sugar with table sugar did not affect the multiplication rate, hence for carrying out micropropagation studies, the sucrose was invariably replaced by less expensive table sugar.

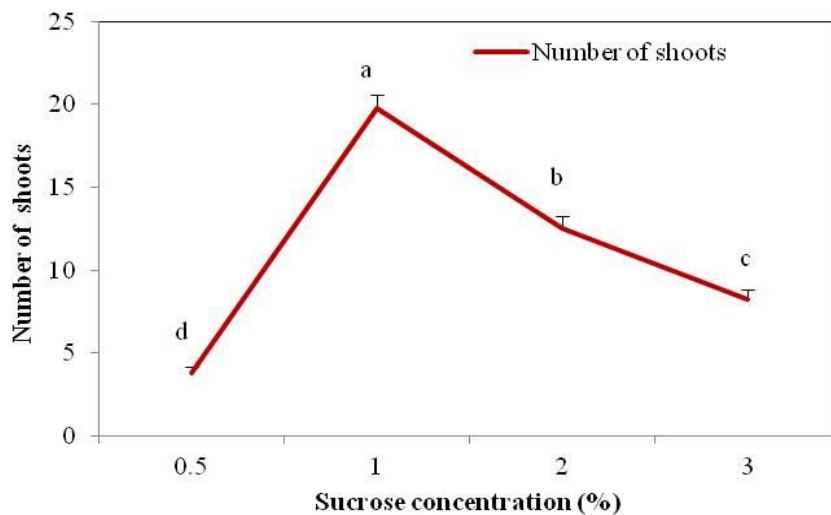


Figure 4.2.2.3 Effect of varying sucrose concentration on shoot proliferation.

4.2.3 Rooting of Microshoots

4.2.3.1 Effect of Auxins

The clumps of 3 to 4 shoots (>2.0 cm) were inoculated onto MS medium for induction of rooting. Since *in vitro* raised shoots failed to root on a hormone free basal medium, use of various auxins like NAA (2.68 to 24.16 μM), IBA (2.45 to 19.66 μM) and IAA (2.85 to 22.84 μM) was attempted. Medium supplemented with NAA (16.11 μM) proved best in term of rooting percentage (76.6%) forming 11.5 ± 1.58 roots per propagule having a root length of 4.09 ± 0.4 cm as depicted in Figures 4.2.3.1 a and b. Prolonged incubation in the rooting medium containing NAA brought about abnormal knotting of roots caused by the inhibitory effect of auxin on their further growth. Hence, the withdrawal of auxin NAA from the

medium was found to be necessary to ensure development of healthy roots. The effects of different concentrations of NAA on rooting have been compiled in [Table 4.2.3.1](#).



Figure 4.2.3.1 a. Root induction on 16.11 μM NAA **Figure 4.2.3.1 b.** Complete plantlets of *B. balcooa*.

Table 4.2.3.1 Effects of NAA on rooting response in *Bambusa balcooa*

PGR	Concentration μM	No of Roots	Root Length (cm)	Rooting %
NAA	0	0.0 ^f \pm 0.00	0.0 ^e \pm 0.00	0
NAA	2.68	0.0 ^f \pm 0.00	0.0 ^e \pm 0.00	0
NAA	5.37	0.0 ^f \pm 0.00	0.0 ^e \pm 0.00	0
NAA	8.05	0.0 ^f \pm 0.00	0.0 ^e \pm 0.00	0
NAA	10.74	0.80 ^f \pm 0.63	1.0 ^d \pm 0.28	5.33
NAA	13.42	1.6 ^e \pm 0.51	2.57 ^c \pm 0.39	10.6
NAA	16.11	11.5 ^a \pm 1.58	4.09 ^a \pm 0.49	76.6
NAA	18.79	5.50 ^b \pm 1.26	2.71 ^c \pm 0.31	36.6
NAA	21.48	4.50 ^c \pm 0.84	2.03 ^b \pm 0.32	30
NAA	24.16	3.60 ^d \pm 1.34	1.18 ^d \pm 0.33	24

Values are mean \pm SD. ^{a,b,c,d,e,f,g} are mean separation within columns by Duncan's multiple range test, $P \leq 0.05$.

4.2.3.2 Effect of Strength of MS Medium on Root Induction

For induction of roots, effects of different strengths i.e. 1/4th strength, 1/2 strength and full strength of MS medium was studied. The medium was supplemented with 16.11 μ M of NAA in all the experiments. It was found that full strength MS medium gave the best rooting response forming 9.50 ± 2.91 roots as shown in Figure 4.2.3.2. There was a decreased rooting when 1/2 and 1/4th strength medium was used forming 1.90 ± 3.14 and 0.80 ± 1.75 roots respectively.

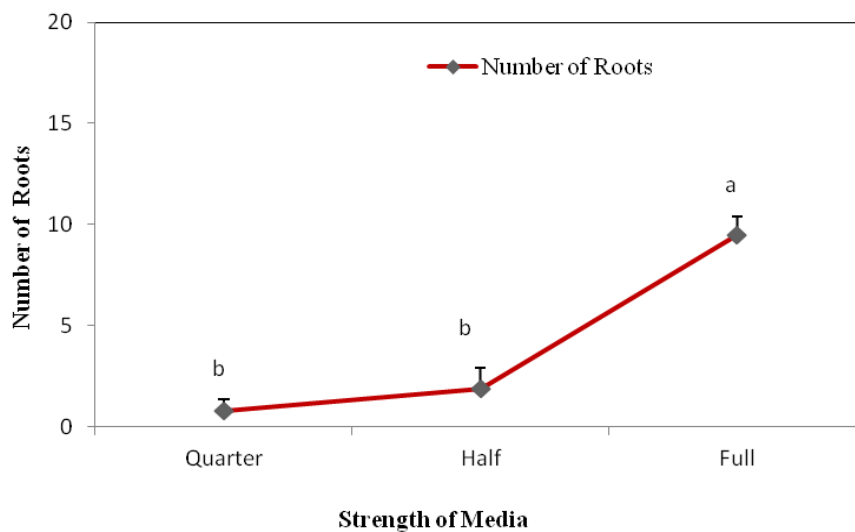


Figure 4.2.3.2. Effect of different strengths of MS medium on rooting.

4.2.4 Hardening and Acclimatization

Plantlets with well developed roots were removed from the culture medium and washed in lukewarm water in a plastic tray (Figure 4.2.4 a). The fully grown plantlets were transplanted to plastic pots containing river bed sand and were covered with transparent covers/jars for maintaining high relative humidity for 2 weeks. After the emergence of new leaves, plants were transferred to the potting mixture containing riverbed sand: soil: farmyard manure in 1:1:1 ratio and shifted to green house (Figures 4.2.4 b and c). During hardening the leaves turned greener and healthier and their lamina expanded significantly. After hardening, the plants were transplanted in the field at the beginning of rainy season in the pits (2ft×2ft×2ft) filled with well rotten farm yard manure (Figure 4.2.4 d). For successful transplanting of plants, a proper inter row and intra row distance was maintained. The plant to plant distance was kept at 6 meters and the growth data was recorded. Plants were watered once in three

days for their proper establishment in the field. The plants were successfully established under field conditions showing high rate of survival (92.5%).



Figure 4.2.4 a. Plantlets washed in luke warm water to remove traces of agar before planting them in pots.
Figure 4.2.4.b. Hardened plantlets in Soil: Sand: FYM ::: 1:1:1



Figure 4.2.4 c Hardened plants after 6 months.



Figure 4.2.4.d. Hardened plants transplanted in the field in the pits (2ft×2ft×2ft).

4.2.5 Evaluation of *In Vitro* Plants and *Ex Vitro* Hardened Plants

A comparative study of various anatomical and biochemical parameters between *in vitro* grown plants and 6 months old *ex vitro* hardened plants was made.

4.2.5.1 Culm Anatomy

An examination of histology of culm was done by light microscopy. T.S of young culm showed the presence of a circular coiled structure i.e. culm sheath. Its structure resembled that of a leaf (Figure 4.2.5.1 a). Vascular bundles were found scattered throughout the stem. Phloem tissue was seen present outside the xylem tissue. Each vascular bundle was enclosed in a bundle sheath of sclerenchymatous cells for providing support (Figure 4.2.5.1 b). The

culms did not show any distinct areas for cortex and pith. Ground tissue present was mainly composed of the parenchymatous cells.

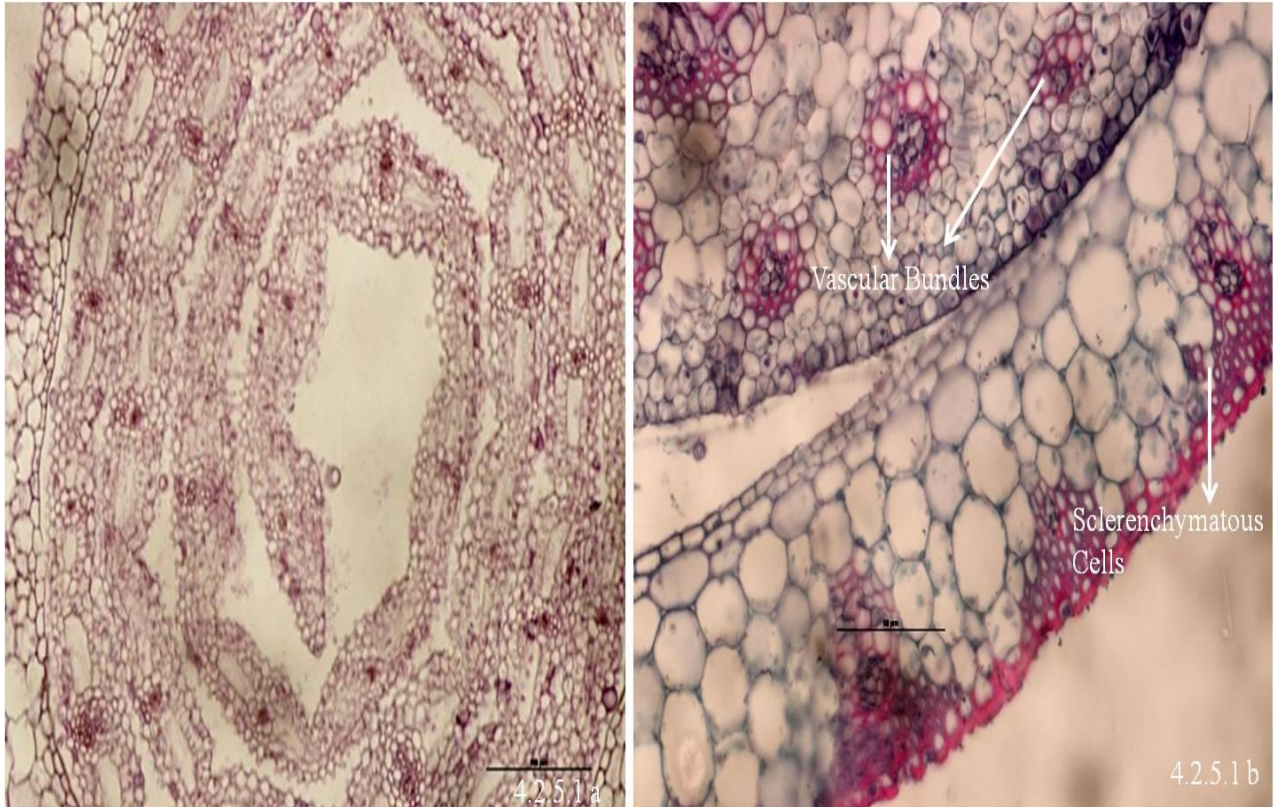


Figure 4.2.5.1 a TS young culm showing coiled culm sheath and also showing numerous vascular bundles (100 X) **Figure 4.2.5.1 b.** Vascular bundles of culm and presence of sclerenchymatous tissue (400X).

4.2.5.2 Leaf Anatomy

The epidermal layer with cuboidal epidermal cells was seen. Specialized epidermal cells called bulliform cells, were also observed which were thin walled, lightly stained and made up of a large central cell surrounded by two small cells. Fusoid cells were seen on both sides of vascular bundles. Trichomes (microhairs) extended out from the surface of the leaf (**Figure 4.2.5.2 a**). They protect leaf from excessive sunlight and loss of water. Vascular bundle consisted of a bundle sheath having compactly arranged cells and numerous sclerenchymatous patches around the sheath. Metaxylem and protoxylem elements for the transport of water and phloem for the transport of food were also seen (**Figure 4.2.5.2 b**).

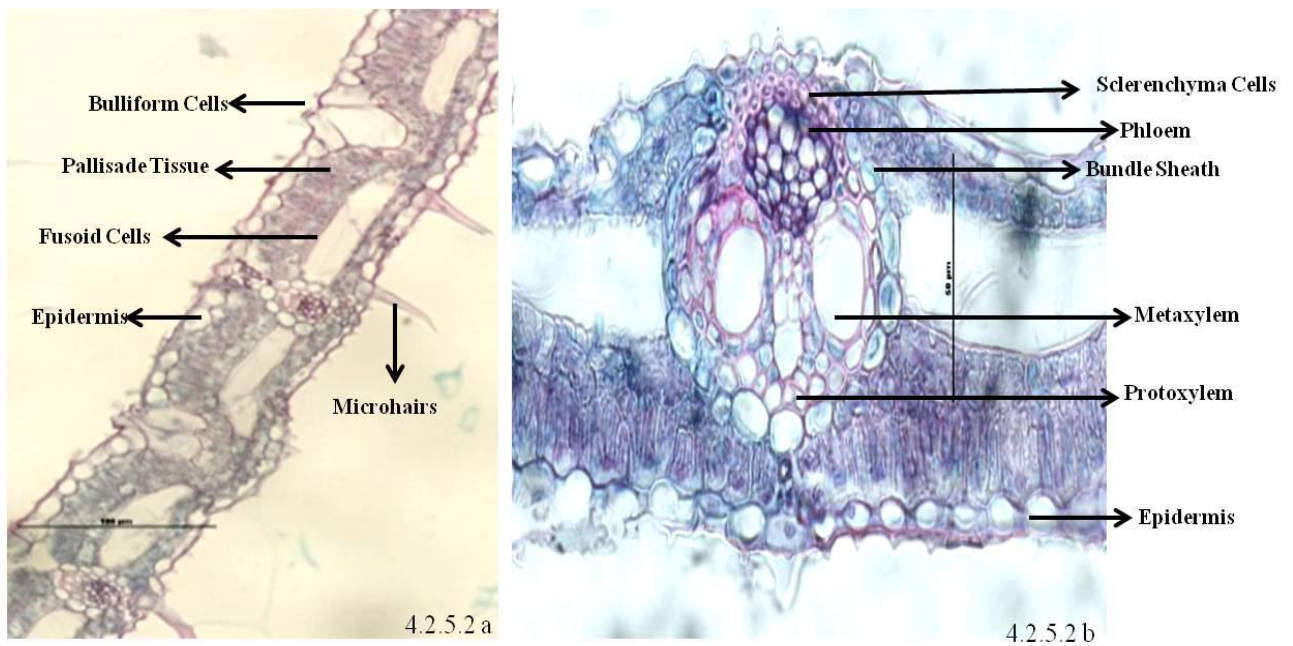


Figure 4.2.5.2 a. Cross section of leaf showing internal anatomy (100 X) **Figure 4.2.5.2 b.** A magnified vascular bundle showing well developed bundle sheath, xylem and phloem (400 X).

4.2.5.3 Root Anatomy

It was a typical monocot root having parenchymatous ground tissue. Xylem elements showed a typical exarch condition. Vascular bundles were numerous, more than six (Figure 4.2.5.3). Xylem and phloem were arranged on different radii. No cambium was present. Epidermis was uniseriate with numerous root hairs.

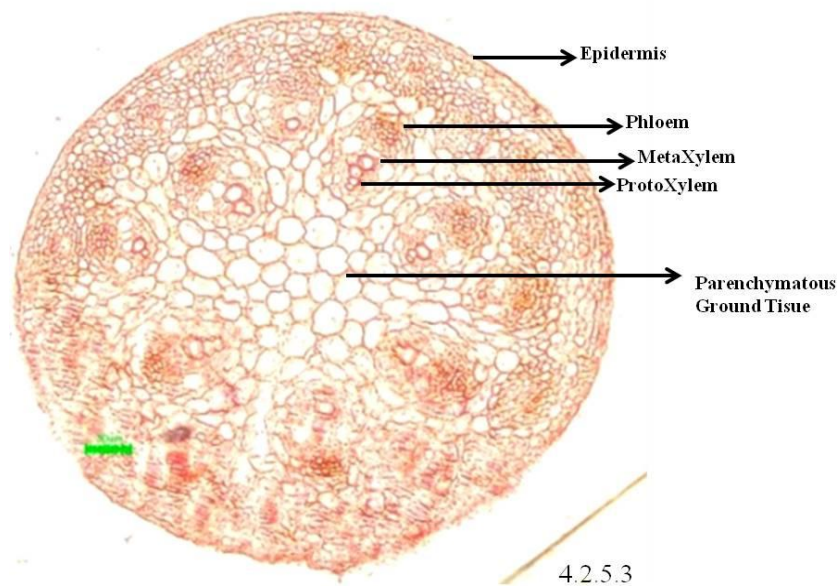
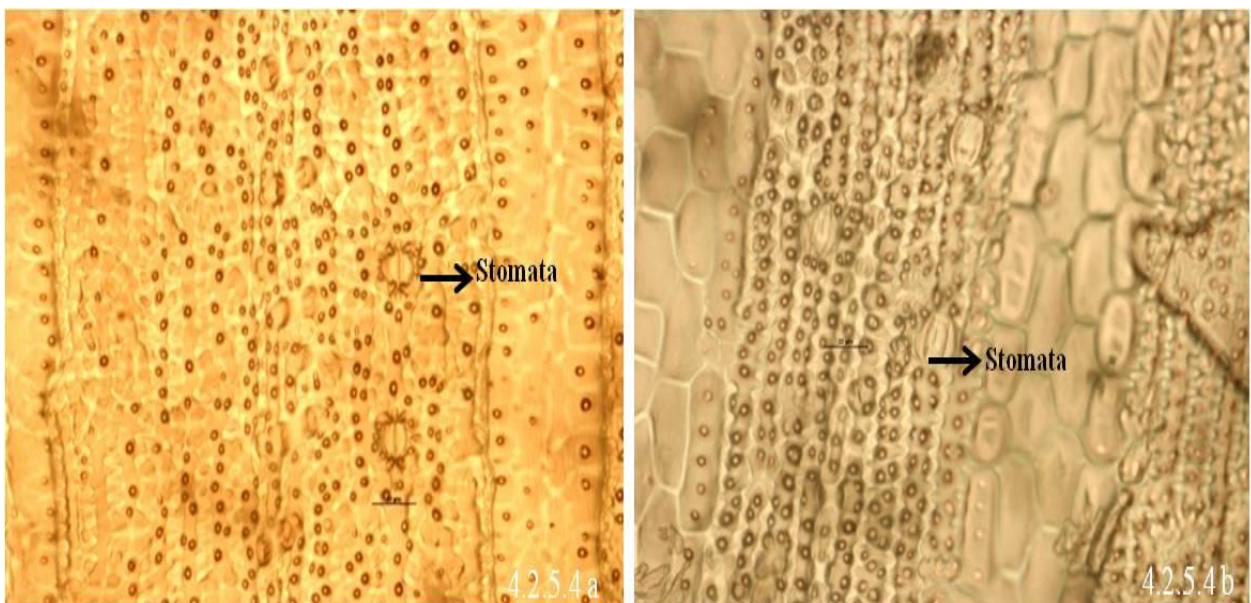


Figure 4.2.5.3 T.S root of *Bambusa balcooa* (400 X).

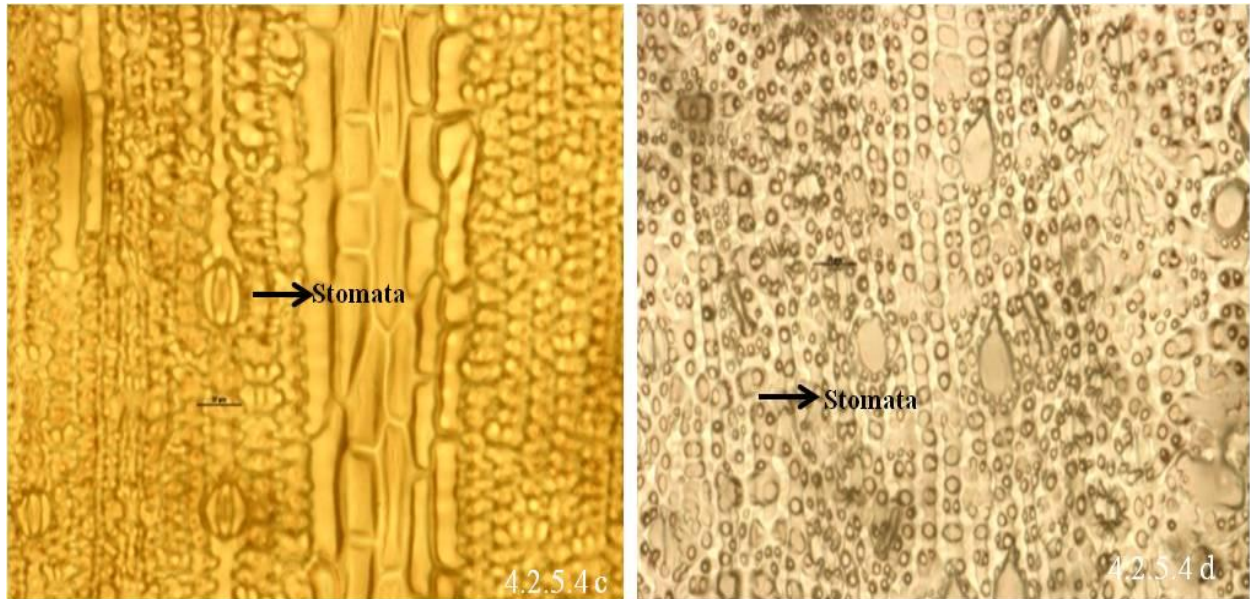
The anatomical details of culm, leaf and root of *in vitro* plants were similar when compared to the field established plants. However, the presence of more lignified tissue in the field plants was a prominent feature.

4.2.5.4 Stomatal Studies (Light Microscopy)

During acclimatization of tissue culture raised plants, changes regarding stomata and photosynthetic machinery were significant and hence, recorded. Stomatal studies were performed in the *in vitro* grown plants and the *ex vitro* acclimatized plants. The results depicted stomata to be more in the *ex vitro* raised plants when compared to the *in vitro* grown plants. *In vitro* grown plants depicted a stomatal density of 18.0 ± 2.64 in $68 \mu\text{m}^2$ on the adaxial surface (Figure 4.2.5.4 a) as compared to the *ex vitro* grown leaf showing a stomatal density of 24.0 ± 2.0 (Figure 4.2.5.4 b). Similarly, stomata on abaxial surface of *in vitro* grown plant had a density of 30.66 ± 4.0 (Figure 4.2.5.4 c) as compared to 41.33 ± 3.21 (Figure 4.2.5.4 d) of *ex vitro* grown plant. It was also observed that the area occupied by the stomata on abaxial side was more as compared to the adaxial side and more number of stomata were found opened. Circular stomata changed shape to elliptical when facing water stress under *ex vitro* conditions. Silica bodies were invariably present scattered in the mesophyll tissue.



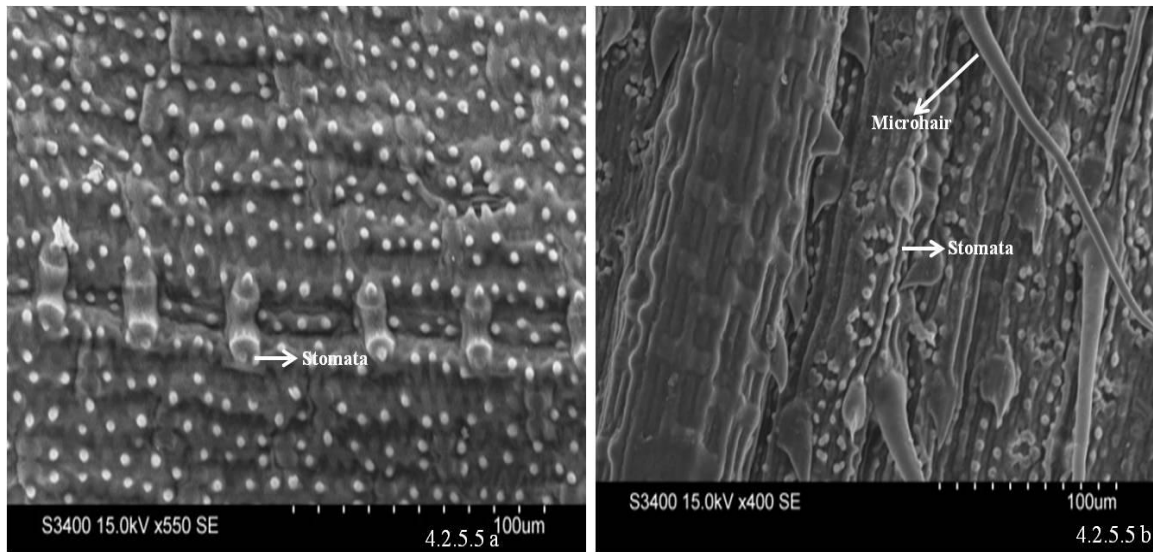
Figures 4.2.5.4 a, b. Stomata on adaxial surface of *in vitro* and *ex vitro* grown leaf respectively.



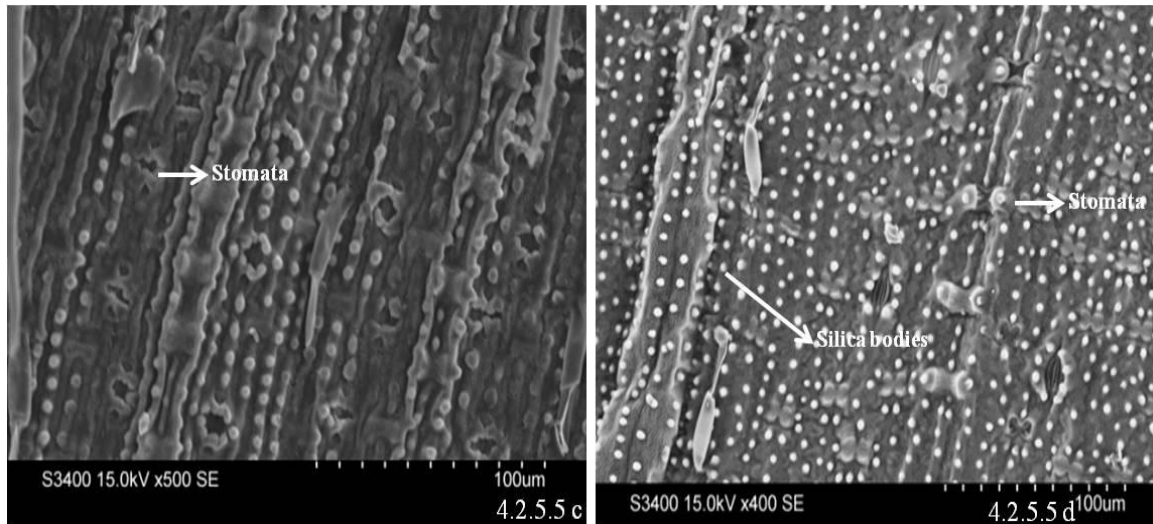
Figures 4.2.5.4 c, d. Stomatal density on abaxial surface of *in vitro* and *ex vitro* grown leaf respectively.

4.2.5.5 Stomatal Studies (Scanning Electron Microscopy)

Samples were selected and cut into smaller size. They were allowed to undergo a short duration pre vacuum process. Samples were then put on sputter coater for coating process before analyzing them under the magnification of SEM. There was a difference in stomatal density of *in vitro* grown and *ex vitro* grown plants. Adaxial surface of *in vitro* leaf showed stomatal density of 32.0 ± 3.0 in $140 \mu\text{m}^2$ (Figure 4.2.5.5 a) as compared to that of *ex vitro* grown leaf showing 42.3 ± 2.51 stomatal density in $140 \mu\text{m}^2$ (Figure 4.2.5.5 b). The abaxial surface of *in vitro* grown leaf has a stomatal density of 48.6 ± 3.5 in $140 \mu\text{m}^2$ (Figure 4.2.5.5 c) as compared to abaxial surface of *ex vitro* grown leaf showing stomatal density of 56.0 ± 2.64 in $140 \mu\text{m}^2$ (Figure 4.2.5.5 d). Hence, the stomatal density was highest on abaxial side of *ex vitro* hardened plants and more number of stomata were found to be open as compared to the ones on the adaxial side. Silica deposition was seen mainly on the abaxial surface of leaf which provides mechanical stability of tissues and protection against microbial infection. Leaves were amphistomatic and microhairs were noticed more on the adaxial surface.



Figures 4.2.5.5 a, b. Scanning electron microscopy (SEM) images showing stomata on adaxial surface of *in vitro* and *ex vitro* grown leaf respectively.



Figures 4.2.5.5 c, d. Scanning electron microscopy (SEM) images showing stomata on abaxial surface of *in vitro* and *ex vitro* grown leaf respectively.

4.2.5.6 Leaf Area Index (LAI)

LAI was used to assess growth and vigour of vegetation. Table 4.2.5.6 depicts that LAI was significantly higher (4.66 ± 0.81) in *ex vitro* hardened plants as compared to the *in vitro* raised plants showing a value of 1.06 ± 0.44 representing physiological differences. LAI values finally affect the net assimilation rates and productivity.

4.2.5.7 Relative Water Content (RWC) and Water Saturation Deficit (WSD)

RWC is one of the important indicator of water status of plant. RWC percentage was found to be higher in the *in vitro* grown plantlets with values of $72.7 \pm 2.3\%$ as compared to the *ex vitro* grown plants showing a value of $67.7 \pm 3.3\%$ (Table 4.2.5.6). The probable reason for these values is due to the growth and development of *in vitro* grown plants under controlled

environmental conditions. There was an inverse relationship of water saturation deficit (WSD) with RWC showing values of $27.3 \pm 2.3\%$ in the *in vitro* plants and a value of $32.3 \pm 3.3\%$ in *ex vitro* grown plants. Low RWC in acclimatized plants indicated that plants were under water stress when shifted from a high RH (85%) to low RH (70%) in green house.

4.2.5.8 Electrolyte Leakage

Electrolyte leakage is a measure of membrane permeability in relation to environmental stresses, growth, development and genotypic variation. The exposure of plants to stressed environment resulted in a significant increase (67.4 ± 2.4) in the percentage of electrolyte leakage (Table 4.2.5.6) as compared to *in vitro* grown plants showing values of $57.7 \pm 2.7\%$. The results of our experiments revealed a relative leakiness of the hardened plants as compared to the plants grown under the controlled conditions.

4.2.5.9 Proline Estimation

Proline accumulates in many plant species in response to environmental stresses, thereby, playing a role in cellular homeostasis. In the present investigation, proline content was found to be more in the *ex vitro* hardened plants (2.47 ± 0.28) $\mu\text{M/g}$ as compared to 1.97 ± 0.08 $\mu\text{M/g}$ in the *in vitro* grown plants as shown in Table 4.2.5.6. Its high value in hardened plants accounts for its role in acclimatization.

4.2.5.10 Lignin Estimation

Lignin being a phenylpropanoid macromolecule is resistant to degradation and provides necessary strength to plant to cope up with environmental stresses. *In vitro* grown plants have lignin content of 17.90 ± 2.16 mg/l as compared to *ex vitro* grown plants having lignin content of 28.74 ± 1.59 mg/l (Table 4.2.5.6). The high level of lignin in hardened plants shows its role in facilitating water transport and providing the line of defense for pathogens, insects and herbivores.

4.2.5.11 Starch Estimation

The analysis of the starch content has shown that *ex vitro* grown plants have a low level i.e. 4.76 ± 0.51 as compared to *in vitro* plants having values of 6.32 ± 0.55 mg g⁻¹ fr.wt. as shown in Table 4.2.5.6. This is attributed to increased growth rate and metabolism of the hardened plants resulting in higher starch consumption for the increase in volume of the plants.

4.2.5.12 Total Soluble Sugars (TSS)

TSS been recorded to be higher in the *in vitro* raised plants i.e. 16.5 ± 0.78 mg g⁻¹ fr.wt. as compared to *ex vitro* raised having a value of 13.38 ± 2.03 mg g⁻¹ fr.wt. (Table 4.2.5.6). This was due to high growth rate in the hardened plants as they were in the log phase of their life

cycle having higher consumption of soluble sugars. These sugars further served as raw material for building other macromolecules for their growth.

4.2.5.13 Chlorophyll Content by Ultrasonics

Chlorophyll content was found to be maximum in the plants growing under the *ex vitro* conditions as compared to the *in vitro* environment. Under *ex vitro* conditions, a higher value (17.25 ± 4.45 mg g⁻¹ fr.wt.) of chlorophyll was recorded as compared to a value of 12.88 ± 0.88 mg/g fr. wt. in the *in vitro* plants when acetone was used as a solvent (Table 4.2.5.13). Increased levels of CO₂ in green house promoted synthesis of chlorophyll and carotenoids. A value of 12.62 ± 2.77 mg g⁻¹ fr.wt. followed by 11.75 ± 2.93 mg g⁻¹ fr.wt. of chlorophyll was recorded when DMSO and DMF were used as solvents and demonstrated that acetone extracted higher content of chlorophyll.

Table 4.2.5.6 Effect of *in vitro* and *ex vitro* environment on some important parameters in *B. balcooa*

Parameters	<i>In vitro</i>	<i>Ex vitro</i>	Remarks
Relative Water Content %	72.7 ^a ± 2.32	67.7 ^b ± 3.33	Plant water status in terms of cellular hydration
Water Saturation Deficit	27.3 ^a ± 2.32	32.3 ^b ± 3.33	Plant water balance
Electrolyte Leakage %	57.7 ^b ± 2.70	67.4 ^a ± 2.41	Cell membrane stability
Leaf Area Index	1.06 ^b ± 0.44	4.6 ^a ± 0.81	More growth and vigor of <i>ex vitro</i> plants
Lignin Content mg/l	17.9 ^b ± 2.16	28.7 ^a ± 1.59	Digestion is inversely co-related with lignification which is a source of fibre in food
TSS mg g ⁻¹ fr.wt.	16.5 ^a ± 0.78	13.3 ^b ± 2.03	TSS indicated utilization of sugars for growth and metabolism
Starch mg g ⁻¹ fr. wt.	6.32 ^a ± 0.55	4.76 ^b ± 0.51	Reserve pool of carbon
Proline Content µM/g	1.97 ^b ± 0.08	2.47 ^a ± 0.28	Redox balance, energy status and cellular homeostasis

Values are mean ± SD. ^{a,b} are mean separation within columns by Duncan's multiple range test, P ≤ 0.05

Table 4.2.5.13 Chlorophyll content in culms of *in vitro* and *ex vitro* grown plants by ultrasonics

Solvents	<i>In vitro</i> (mg/g fr.wt.)	<i>Ex vitro</i> (mg/g fr.wt.)	Remarks
Acetone	12.88 ^a ± 0.88	17.25 ^b ± 4.45	More stable and less abrasive solvent
DMSO	10.48 ^b ± 1.65	12.62 ^b ± 2.77	Less toxic than DMF
DMF	7.75 ^c ± 0.84	11.75 ^a ± 2.93	More viscous, toxic and less stable

Values are mean ± SD. ^{a,b,c} are mean separation within columns by Duncan's multiple range test, P ≤ 0.05

4.3 Micropropagation studies in *Bambusa bambos*

4.3.1 Initiation of Aseptic Cultures

4.3.1.1 Choice of Explants

Different explants like nodal segments from tertiary branches, leaves and young buds were tested for their suitability for regeneration potential. Only nodal segments were found to be suitable for culture initiation. Leaf explants failed to respond to any of the media combinations tried while young buds from main culm failed to sprout and proliferate. Single node cuttings from second and third internode of tertiary branches of a 5 year old vegetatively propagated healthy plant of *B. bambos* were collected and used for initiating *in vitro* cultures. Prior to surface sterilization, leaf sheath that enveloped the axillary bud and a part of the upper internode were removed. Fungal contamination was found to be the major bottleneck in initiating aseptic cultures, so a suitable sterilization procedure was adopted involving use of bavistin (0.25%) along with streptomycin sulphate (0.02%) and tetracycline (0.02%) for 30 minutes followed by treatment with sodium hypochlorite (15%) for 8 minutes. The explants were then treated with HgCl₂ (0.1%) for 8 minutes before inoculating on to MS medium for sprouting. The ends of explants were trimmed to remove the dead portions and then placed vertically in the culture medium for the initiation of cultures.

4.3.2 Nodal Explants

4.3.2.1 Seasons vs Culture Initiation

The collection time of explant for culture initiation greatly influenced the frequency of bud break and number of shoots produced per explant. Explants collected during spring in the months of February to April gave best response in terms of increased bud break (93%), early shoot initiation and decreased contamination. Rainy season (June to August) depicted 97% bud sprouting on basal MS medium but was not a preferred season as it had a direct influence on contamination rate (95%) and survival percentage of explants. Summers and winters were the least preferred seasons as the percentage of bud break was very low being 37 and 50% respectively. This may be due to less vigorous growth of mother plant in these seasons having direct bearing on the morphogenetic potential of the axillary buds under *in vitro* conditions. Variations in bud break percentage in *B. bambos* during the year is shown in the [Figure](#)

4.3.2.1

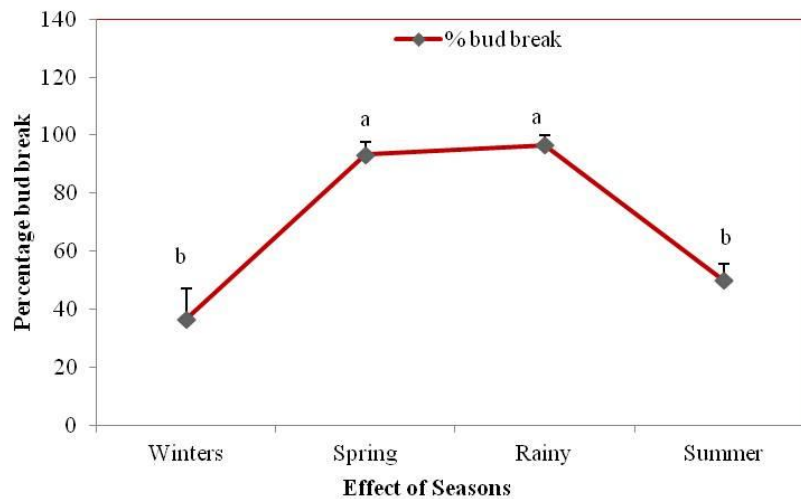


Figure 4.3.2.1. Effect of seasons on the frequency of bud break.

4.3.2.2 Effect of Explant Size

The size of the explant was another parameter studied for bud break efficiency during the initiation phase of the cultures. It was observed that the larger explants (25 mm) took the least time for sprouting which started after 10 days of culture (Figure 4.3.2.2). The small sized explants (5 mm) took much longer time i.e. 23 days for sprouting. The larger explants during the initiation phase of cultures survived the treatment with sterilants in a better way than the small sized explants and had more endogenous nutrients to support the growth under *in vitro* environment.

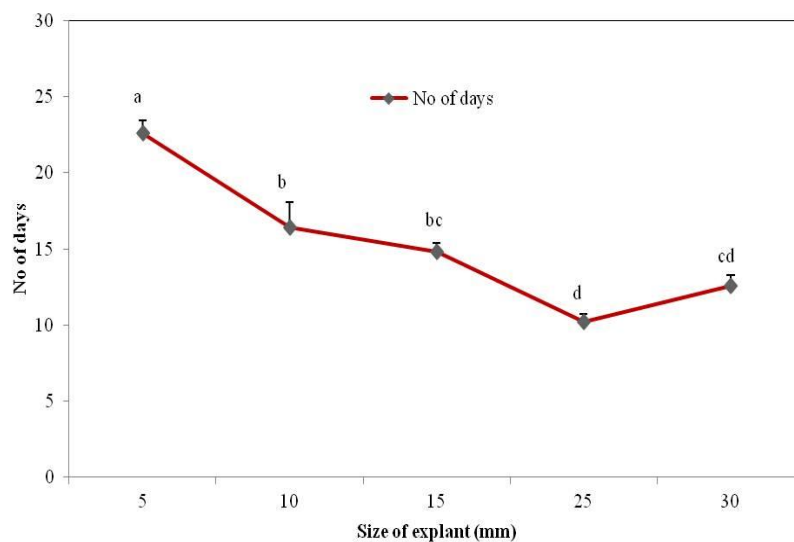


Figure 4.3.2.2. Effect of explant size on bud break.

4.3.2.3 Standardization of Shoot Multiplication Medium

For bud sprouting, the nodal explants were initially cultured on MS basal medium without any growth regulator. Bud break occurred after one week of planting forming 2-3 shoots which however did not multiply further (Figures 4.3.2.3 a and b). For further shoot proliferation, the sprouted buds were excised from the mother stump and placed on cytokinin supplemented MS medium. Effects of cytokinins like BAP (1.1 to 17.6 μM) and Kn (1.16 to 9.3 μM) on shoot proliferation were evaluated in terms of number of shoots formed, shoot length and number of leaves. Out of different concentrations of cytokinins tested, 4.4 μM of BAP alongwith 1.16 μM of Kn gave best proliferation rate forming 8-10 shoots after 3 weeks (Figures 4.3.2.3 c) and nearly 21.70 ± 2.40 shoots having a shoot height of 5.76 ± 0.24 cm were formed after 6 weeks (Figures 4.3.2.3 d). Higher concentration of BAP (8.8 μM) not only lowered the multiplication rate but also resulted in stunted shoots. Likewise an increase in the concentration of Kn considerably reduced the number of shoots formed. An increased rate of multiplication was reported when the shoot clusters obtained at the end of each multiplication cycle were sub-divided into smaller cluster of 3-4 shoots and replanted on the fresh multiplication medium at regular intervals of 4-5 weeks. Table 4.3.2.3 shows the effects of different concentrations and combinations of cytokinins on multiple shoot proliferation in *B. bambos*.

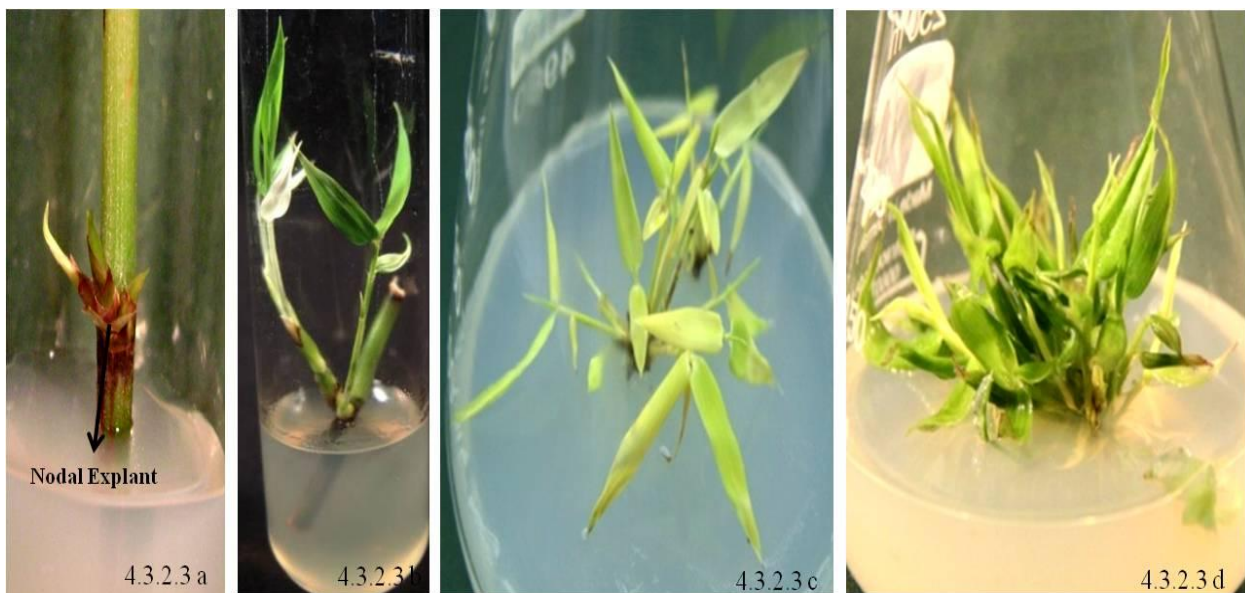


Figure 4.3.2.3 a-b Bud break on basal MS medium **Figure 4.3.2.3 c** Initiation of multiple shoots on MS supplemented with 4.4 μM BAP and 1.16 μM Kn after 3 weeks. **Figure 4.3.2.3 d** Proliferation of numerous shoots observed after 6 weeks on the same medium.

Table 4.3.2.3 Effect of BAP and Kn on Shoot Proliferation in *B. bambos*

BAP+ Kn (μM)	No of shoots	Shoot height (cm)	Total no of leaves
0.0+ 0.46	4.0 ^{hi} \pm 1.41	1.75 ^{hi} \pm 0.44	11.2 ^g \pm 2.34
0.0+ 1.16	3.20 ^{ij} \pm 1.13	1.08 ^j \pm 0.35	6.30 ⁱ \pm 2.4
0.0+ 2.3	8.30 ^g \pm 2.00	1.03 \pm 0.20	8.8 ^h \pm 1.39
0.0+ 4.65	5.30 ^h \pm 0.94	0.80 ^{ij} \pm 0.26	7.7 ^{hi} \pm 1.15
0.0+ 6.85	3.60 ⁱ \pm 0.96	0.69 ^j \pm 0.22	6.3 ⁱ \pm 1.88
0.0+ 9.3	1.80 ^j \pm 0.78	0.61 ^j \pm 0.24	5.8 ⁱ \pm 1.68
2.2+ 0.0	12.80 ^{ef} \pm 1.68	2.77 ^g \pm 0.21	14.1 ^{eb} \pm 2.28
2.2+ 0.46	13.80 ^e \pm 2.34	3.30 ^f \pm 0.36	18.3 ^e \pm 1.63
2.2+ 1.16	16.10 ^c \pm 1.59	3.87 ^{de} \pm 0.33	23.8 ^c \pm 2.25
2.2+ 2.3	12.60 ^{ef} \pm 1.83	3.73 ^{de} \pm 0.35	23.6 ^c \pm 3.30
4.4+ 0.0	15.50 ^{cd} \pm 3.24	3.31 ^f \pm 0.39	21.8 ^d \pm 2.04
4.4+ 0.46	19.20 ^b \pm 1.61	4.60 ^c \pm 0.37	26.8 ^b \pm 3.30
4.4+ 1.16	21.70 ^a \pm 2.40	5.76 ^a \pm 0.24	28.6 ^a \pm 2.01
4.4+ 2.3	17.80 ^b \pm 1.31	5.03 ^b \pm 0.33	18.2 ^e \pm 1.93
8.8+ 0.0	15.7 ^c \pm 1.76	4.03 ^d \pm 0.38	17.9 ^e \pm 1.72
8.8+ 0.46	14.0 ^{de} \pm 1.63	3.55 ^{ef} \pm 0.32	11.8 ^e \pm 1.31
8.8+ 1.16	11.7 ^f \pm 1.88	2.87 ^g \pm 0.28	14.2 ^f \pm 2.44
8.8+ 2.3	8.0 ^g \pm 2.2	1.60 ⁱ \pm 0.34	11.9 ^g \pm 1.66

Values are mean \pm SD. ^{a,b,c,d,e,f,g} are mean separation within columns by Duncan's multiple range test, $P \leq 0.05$

4.3.2.4 Effect of Sucrose Concentrations on Shoot Multiplication

Serving as a carbohydrate source, sucrose also regulates osmolarity of the culture medium and helps during morphogenesis. To study the effects of different concentrations of sucrose on shoot multiplication, three to four shoots/clump were cultured on MS medium containing BAP (4.4 μM) and Kn (1.16 μM) alongwith different concentrations of sucrose. Out of different concentrations (0.5 to 3.5%) tried, 2% sucrose was found to be most suitable forming on an average 21.1 shoots after 4-5 weeks of culture (Figure 4.3.2.4). The results indicated that proliferation of shoots decreased when the concentration of sugar was increased to 3%. Graded sucrose as well as commercial table sugar were used and both gave an equally good response for proliferation. Hence, commercial table sugar being cheaper was preferred over sucrose for carrying out micropropagation protocol.

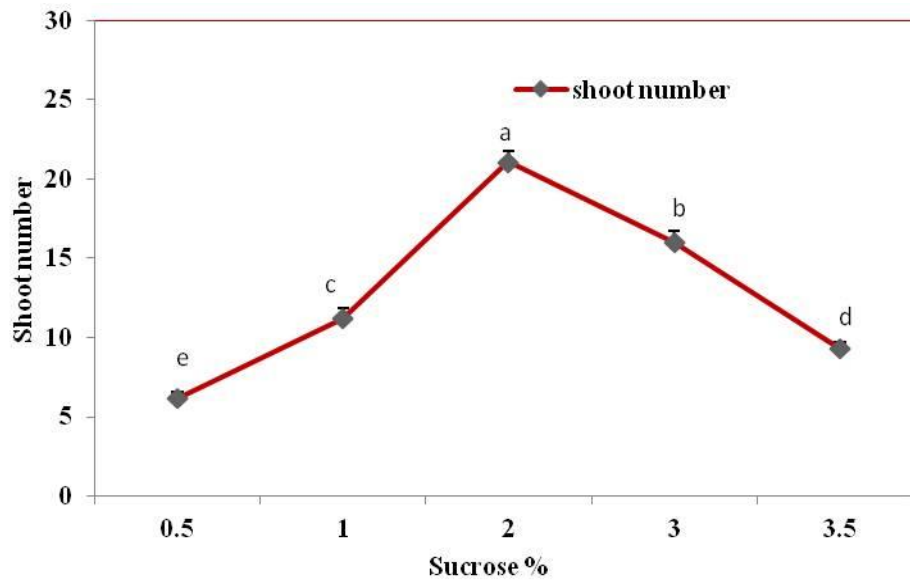


Figure 4.3.2.4. Effect of sucrose concentrations on axillary shoots proliferation

4.3.3 Rooting of Microshoots

In general, rooting is a main bottleneck while carrying out *in vitro* multiplication of bamboos and has been a major problem to be worked out in this particular species as well. It was observed that rooting was more effectively induced when clusters of shoots rather than individual shoot were used. The clumps of 3 to 4 shoots were inoculated on full MS, ½ MS and ¼ MS medium supplemented with different concentrations and combinations of auxins. Half strength MS medium fortified with 9.80 µM of IBA gave the best response for induction of roots with a root length of 7.04 ± 0.79 cm within 30 to 45 days (Figures 4.3.3 a and b) in 60% of cultures. Out of the various concentrations of NAA (2.68 to 24.16 µM) tried, NAA at 5.37 µM produced rooting in 48% cultures. It was observed that the addition of coumarin in the root inducing medium further improved the rooting response. An initial pulse treatment of coumarin (9 mg/l) for 10 days and then shifting the cultures to coumarin free medium enhanced the rooting in all the combinations. Although auxins were essential for the induction of roots, but transfer of *in vitro* rooted shoots to basal MS medium after 30-45 days further improved rooting. Shoots failed to root on MS medium supplemented with IAA even after 45 days of culture. Hence, NAA and IBA proved to be effective for providing initial stimulus for *in vitro* rooting while advanced root growth was noticed only upon transfer to auxin free medium. Table 4.3.3 shows the effects of different concentrations of IBA and NAA on rooting of *in vitro* plants.



Figure 4.3.3 a. Induction of roots on 9.80 μM of IBA.
Figure 4.3.3 b. A complete plantlet formed.

Table 4.3.3 Effects of Auxins on Rooting in *B. bambos*

PGR	Concentration μM	No. of roots	Root length (cm)	Rooting %
IBA	2.45	0.0 ^g \pm 0.0	0.0 ^d \pm 0.0	0
IBA	4.90	0.0 ^g \pm 0.0	0.0 ^d \pm 0.0	0
IBA	9.80	7.8 ^a \pm 1.3	7.0 ^a \pm 0.7	60
IBA	14.76	2.8 ^{de} \pm 1.3	4.5 ^{bc} \pm 0.7	32
IBA	19.68	0.8 ^{fg} \pm 0.8	1.7 ^e \pm 2.3	8
IBA	24.5	0.6 ^{fg} \pm 0.5	0.8 ^{de} \pm 1.7	4
NAA	2.68	2.4 ^{de} \pm 1.1	1.6 ^e \pm 0.5	18
NAA	5.37	6.4 ^b \pm 1.1	5.6 ^{ab} \pm 0.5	48
NAA	10.74	5.0 ^c \pm 1.5	5.7 ^{ab} \pm 0.5	44
NAA	16.11	3.8 ^{cd} \pm 1.6	4.0 ^c \pm 0.3	32
NAA	21.48	1.8 ^{ef} \pm 0.8	3.3 ^c \pm 0.5	28
NAA	24.16	1.6 ^{ef} \pm 0.8	1.5 ^e \pm 1.4	12

Values are mean \pm SD. ^{a,b,c,d,e,f,g} are mean separation within columns by Duncan's multiple range test, $P \leq 0.05$

4.3.4 Hardening and Acclimatization

Transfer of plantlets and acclimatization to *ex vitro* environment is the final and the most important step for any successful micropropagation protocol. The *in vitro* raised plantlets were taken out of the culture jars and washed thoroughly under running tap water to remove the adhered agar from the roots. Initially the plants were placed inside a growth room for acclimatization in small pots containing moist river bed sand. After 14 days, all the plantlets were transferred to polythene bags (7''x 5'') containing a potting mixture of soil, sand and farmyard manure in equal proportion. Transferred plants exhibited normal growth when shifted to green house (Figure 4.3.4 a). The plants were initially placed close to the cooling pads (relative humidity 80-85%), gradually shifted away from the pads towards the exhaust fans over a period of 1 week. The foliage was sprayed with Hoagland solution after every 7 days. Thereafter, the plants were nurtured inside a polyhouse for a long period to tide over the unfavourable winter conditions outside. Finally the plants were transplanted in the field to pits (2ft×2ft×2ft) at a plant to plant and row to row distance of 6 meters in the rainy season. The plants were successfully established under the field conditions with 80% survivability (Figure 4.3.4 b).



Figure 4.3.4 a Six months-old hardened plant. **Figure 4.3.4 b** Six months-old hardened plant in field.

4.3.5 Callus Studies in *Bambusa bambos*

4.3.5.1 Induction and Proliferation of Callus

For induction of callus, internodal segments (0.5-1cm), leaves and leaf bases from *in vitro* raised shoots were taken and inoculated onto MS medium supplemented with different auxins like 2,4-D (1.13 to 18.2 μM) and NAA (1.34 to 21.48 μM) either alone or in conjunction with each other. 4.53 μM of 2,4-D and 5.37 μM of NAA alongwith 3% sucrose were found to be the best for the initiation of callus when incubated in the dark. Callusing started at the cut ends of the internodal explants after 15 days (Figure 4.3.5.1 a) and proliferated further forming a mass of whitish yellow callus after 4 weeks (Figure 4.3.5.1 b). Both nodular (whitish yellow and compact) and mucilagenous (translucent and jelly like) calli were observed (Figure 4.3.5.1 c) but only the nodular and the compact callus was found to be organogenetic and taken for the further experiments. Effects of various concentrations of 2, 4-D and NAA on callus induction are shown in the Table 4.3.5.1. Leaves and leaf bases were also used as explants to induce callus but did not respond on any of the combination of the growth regulators tried.

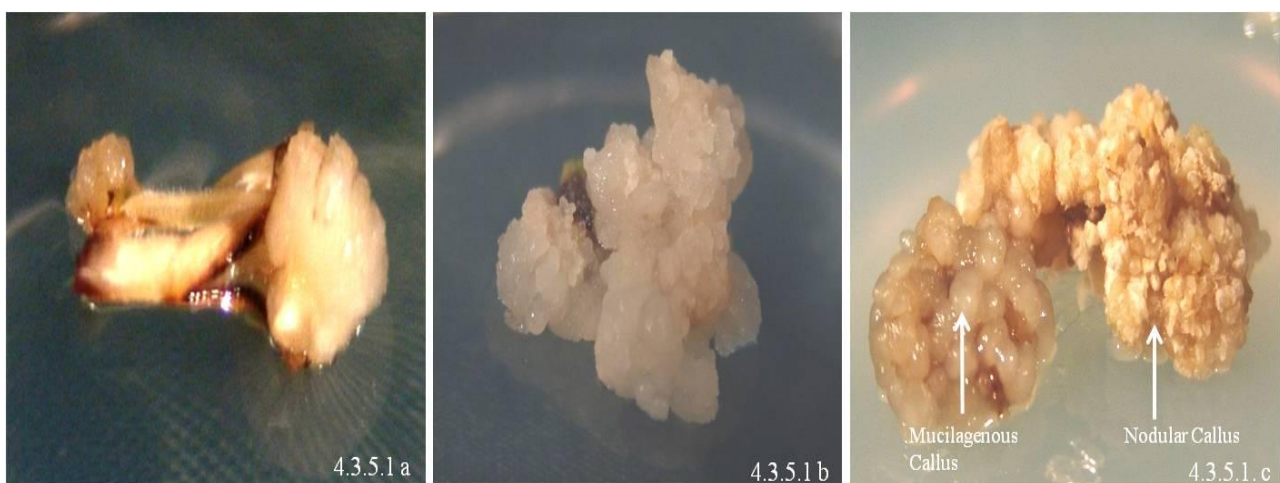


Figure 4.3.5.1 a. Initiation of callus on 4.53 μM of 2, 4-D and 5.37 μM of NAA. **Figure 4.3.5.1 b.** Proliferation of callus after 4 weeks. **Figure 4.3.5.1 c.** Callus of both mucilagenous and nodular type observed on the initiation medium.

Table 4.3.5.1 Effects of Various Auxins on Callus Induction

S.No	Media Composition	Concentration (μM)	% Callus Induction	Days
1	MS	-	0	-
2	MS+2,4-D	1.13	50	30
3	MS+ 2,4-D	2.26	55	30
4	MS+ 2,4-D	4.53	70	25
5	MS+ 2,4-D	9.06	50	30
6	MS+ 2,4-D	13.59	45	35
7	MS+ 2,4-D	18.20	30	35
8	MS+NAA	2.68	0	-
9	MS+NAA	5.37	0	-
10	MS+ 2,4-D+ NAA	4.53+ 5.37	90	25
11	MS+ 2,4-D +NAA	9.06+ 10.74	45	25

4.3.5.2 Rhizogenesis

When callus was shifted to lower concentration of 2, 4-D ($2.26 \mu\text{M}$) in conjunction with NAA ($1.14 \mu\text{M}$), 40% callus lumps showed development of roots. Initially a few roots were formed which were short and swollen (Figure 4.3.5.2 a) but with further proliferation of callus more and more roots were formed which further increased in length (Figure 4.3.5.2 b).

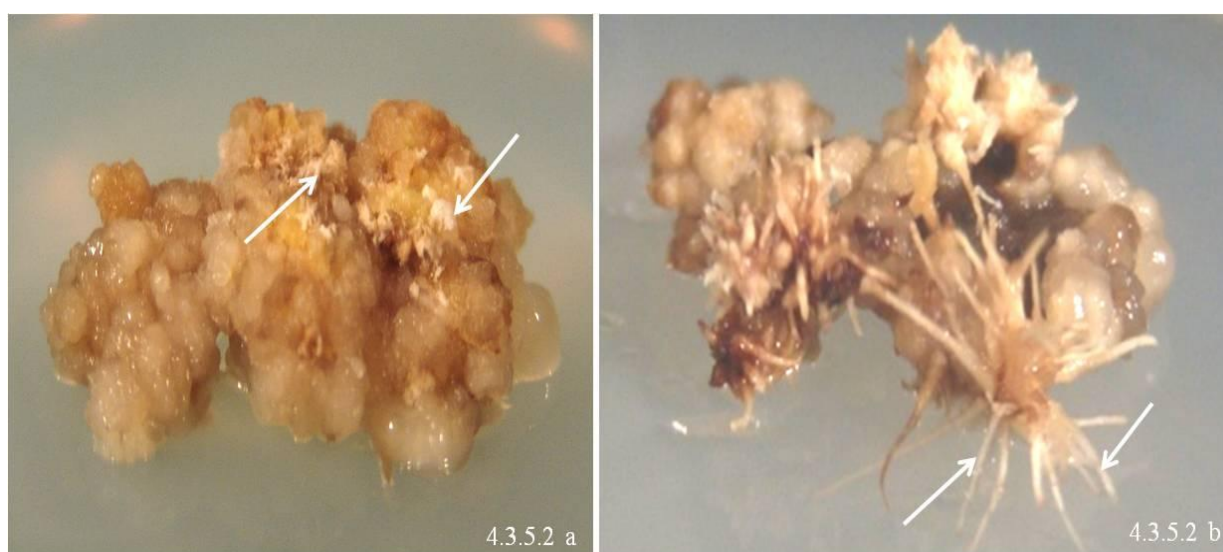


Figure 4.3.5.2 a. Emergence of short swollen roots from nodular callus Figure 4.3.5.2 b. Formation of numerous roots on $2.26 \mu\text{M}$ of 2,4-D and $1.14 \mu\text{M}$ of NAA after 30 days.

4.3.5.3 Caulogenesis

Differentiation of shoot buds from the callus occurred after 6 weeks on MS medium containing $4.53 \mu\text{M}$ of 2, 4-D and $5.37 \mu\text{M}$ of NAA (Figure 4.3.5.3 a). Shoot bud elongation started after 10 days on the same medium but their further growth was arrested and buds started turning brown. Hence, they were shifted to MS medium supplemented with low

concentration of BAP (2.2 μM) alongwith 4.53 μM of 2,4-D where further elongation of shoot buds occurred forming shoots with leaves after 1 month as shown in [Figure 4.3.5.3 b](#). The frequency of shoot bud differentiation was very low being 20% only. Addition of other cytokinins like Zeatin (2.28 -9.12 μM), TDZ (2.27-9.08 μM) and 2-ip (2.46 – 9.84 μM) in the medium did not prove effective for shoot bud induction or embryogenesis and invariably resulted in the browning of callus. Incorporation of any additional auxins (NAA, IBA, IAA) also turned the callus necrotic. Hence, the embryogenic potential and regeneration capacity of the callus still needs to be worked out further.

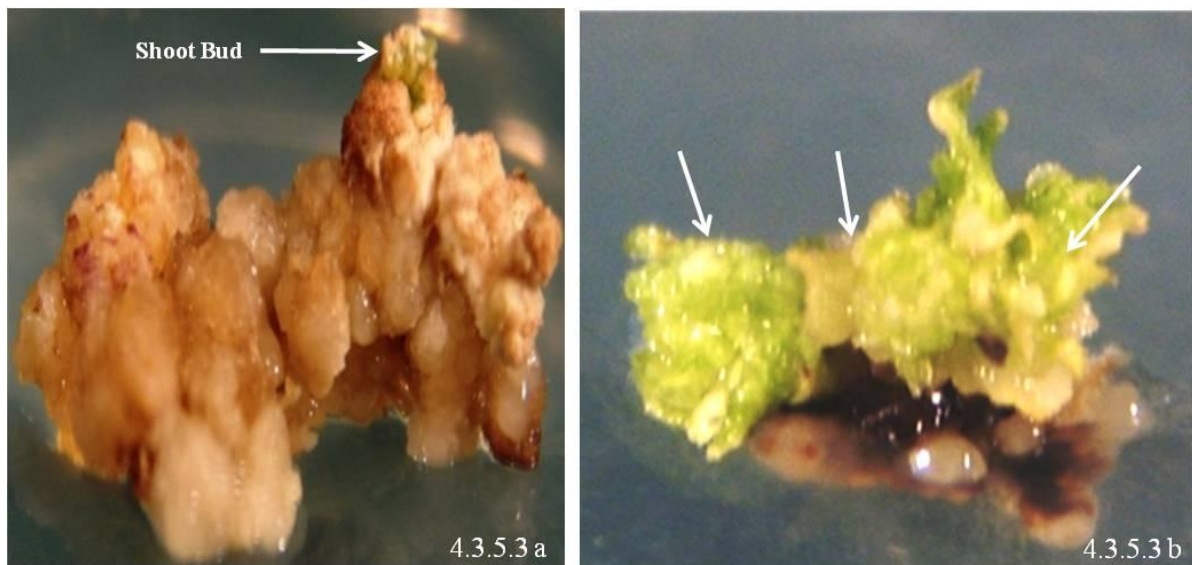


Figure 4.3.5.3 a. Nodular callus showing differentiation of a shoot bud on MS medium containing 2,4-D (4.53 μM) and NAA (5.37 μM). **Figure 4.3.5.3 b.** Numerous shoot buds formed on 4.53 μM of 2, 4-D and 2.2 μM of BAP after 1 month of culture.

4.3.5.4 Histological Studies of Callus (Light Microscopy)

The histology of the nodular callus revealed the organization of cells in a definite pattern ([Figure 4.3.5.4 a](#)). During the active period of growth of the callus, it turned organogenetic and development of meristemoids took place from the cells which turned meristematic and could be recognized by dense cytoplasm and dividing cells ([Figure 4.3.5.4 b](#)). Histological observations further revealed the development of shoot primordia from calli in due course of time (45 days) which subsequently developed into shoot buds having distinct shoot apical meristems ([Figure 4.3.5.4 c](#)).

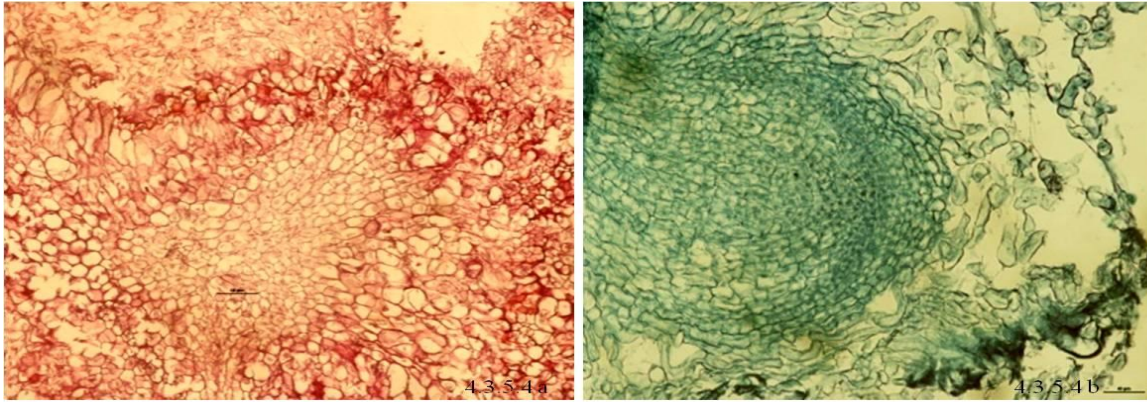


Figure 4.3.5.4 a. Histology of nodular callus cells showing flow of cells in a definite pattern (400 X)

Figure 4.3.5.4 b. Meristemoid formation from nodular callus (400 X).

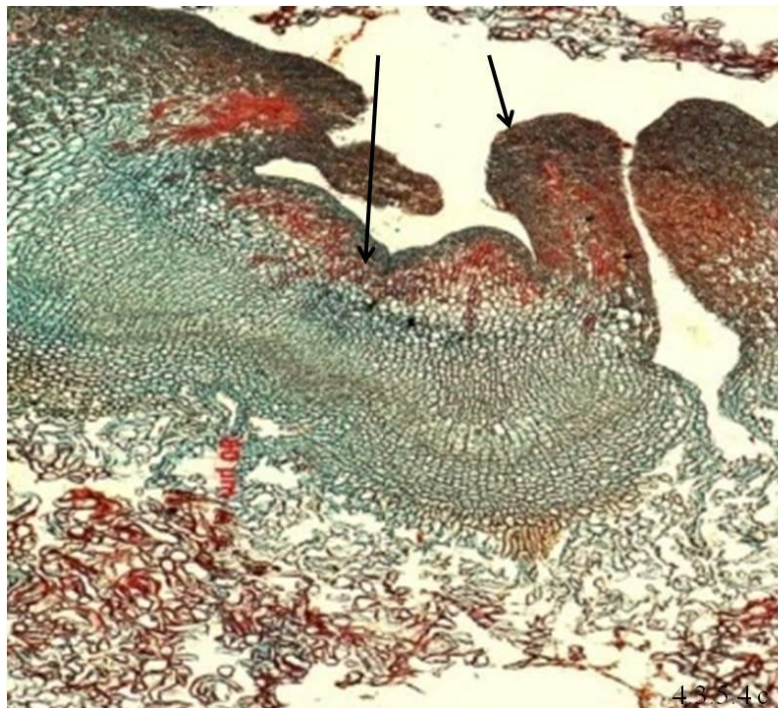


Figure 4.3.5.4 c. Histology of callus showing formation of shoot buds confirming Caulogenesis (400 X).

4.3.5.5 Histological Studies (Scanning Electron Microscopy)

To understand more about the differentiation phenomena, Scanning Electron Microscopy (SEM) was performed at different stages of callus development. SEM was performed using 15, 30, 45 days old callus tissues. The cells of the nodular callus were seen as outgrowths from the surface (Figure 4.3.5.5 a). The shoot primordia were seen growing as outgrowths on the surface of the callus after 4 weeks (Figure 4.3.5.5 b) which eventually developed into shoot buds. The cells of shoot bud appeared as angular and elongated.

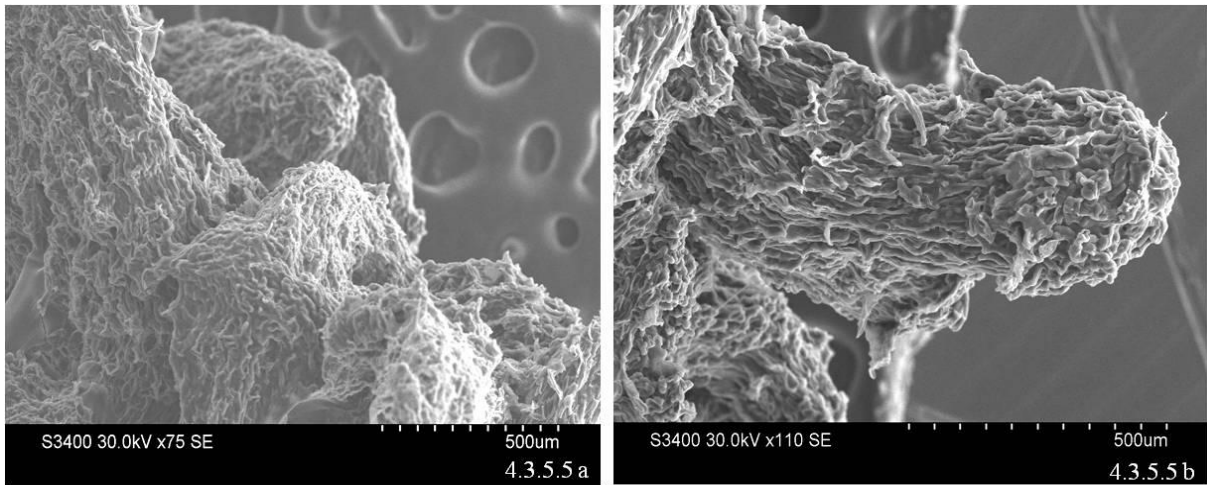


Figure 4.3.5.5 a. Cells of nodular callus. **Figure 4.3.5.5 b.** SEM images showing emergence of shoot primordia from the surface of callus.

4.3.6 Evaluation of *In Vitro* Raised Plants and *Ex Vitro* Plants

For the evaluation of different parameters such as anatomy and biochemical studies, 6 months old acclimatized field plants and 6 weeks old *in vitro* grown plants were taken as the experimental material.

4.3.6.1 Culm Anatomy

T.S. of the culm showed the ground tissue consisting of parenchyma cells with starch grains. It had many collateral, closed vascular bundles which were more numerous and smaller at the periphery (**Figure 4.3.6.1 a**), whereas, the central portion depicted larger and fewer bundles. Each vascular bundle consisted of xylem having protoxylem and metaxylem elements. Circular or slightly elliptical vessels were seen in xylem. Phloem was present on the outer side. Sclerenchymatous tissue was seen present as caps of vascular bundles (**Figure 4.3.6.1 b**).

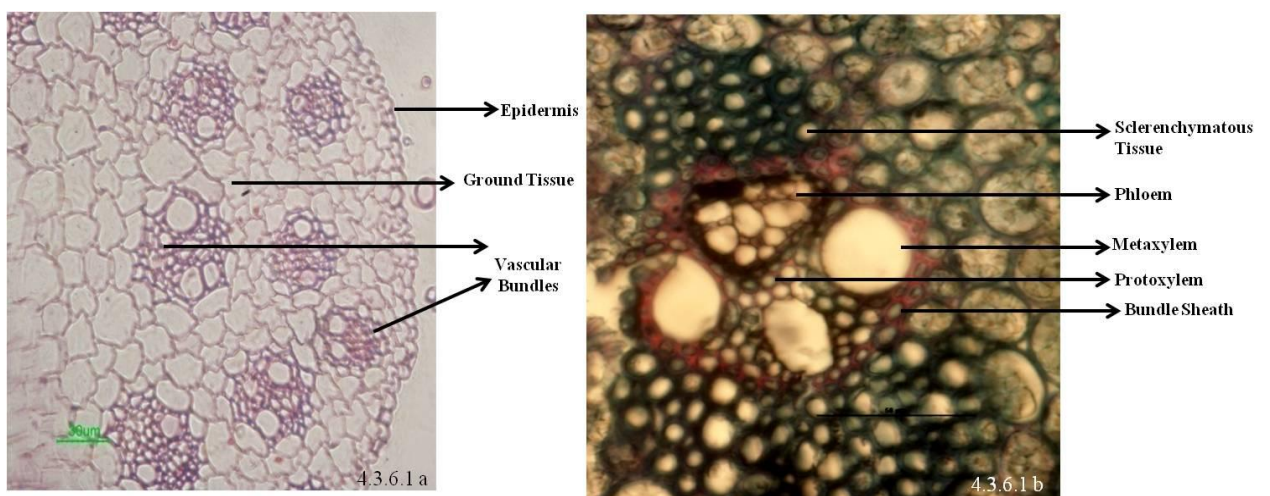


Figure 4.3.6.1 a. T.S. of culm of *B. bambos* showing vascular bundles embedded in ground tissue (100 X). **Figure 4.3.6.1 b.** A collateral vascular bundle showing xylem, phloem and sclerenchymatous bundle sheath (400 X).

4.3.6.2 Leaf Anatomy

Cross section of leaf showed the presence of palisade tissue bounded by upper and lower epidermis (Figure 4.3.6.2 a). One of the evident features of the leaf was the presence of fan shaped bulliform cells which mainly function in the rolling of leaves when plant faces water stress. The vascular system consists of varied size collateral bundles located among fusoid cells (Figure 4.3.6.2 b). A group of sclerenchymatous cells interconnecting vascular bundles with upper and lower epidermis were seen in the regions of midrib. The vascular system organization was observed to be similar all along the midrib except some minor variations in the amount of sclerenchymatous tissue.

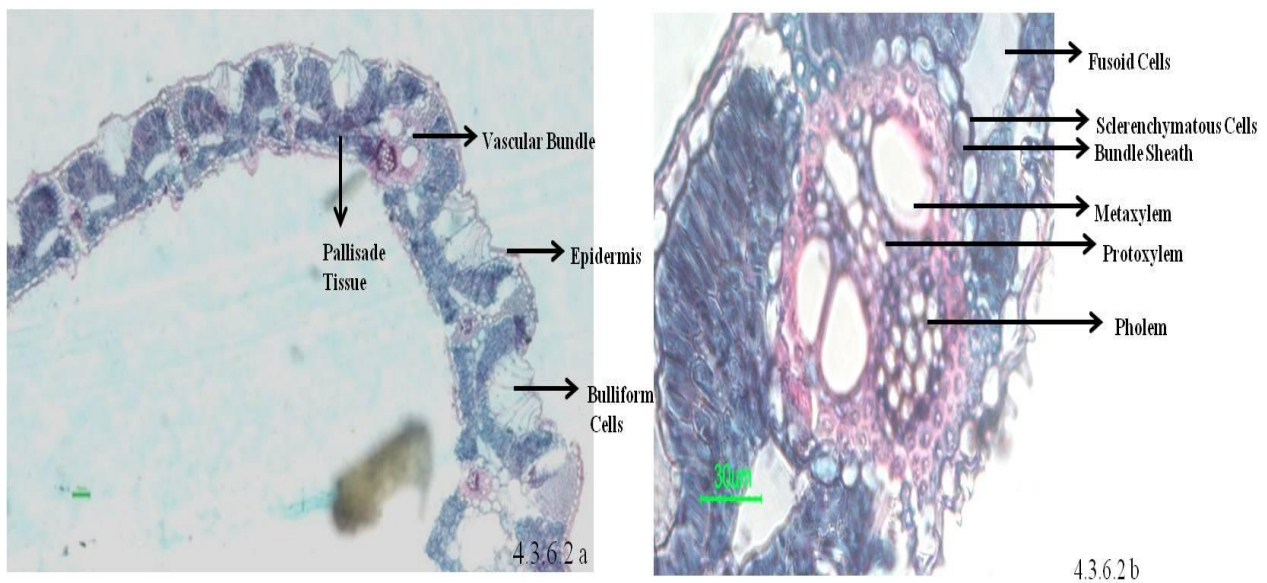


Figure 4.3.6.2 a. Cross section of leaf showing internal anatomy (100 X). **Figure 4.3.6.2 b.** Vascular bundle in leaf of *B. bambos* (400 X).

4.3.6.3 Root Anatomy

T.S. of root showed vascular bundles arranged in the form of a ring around the pith. A layer of endodermis separated the parenchymatous cortex. Vascular bundles were closed and collateral (Figure 4.3.6.3). The amount of vascular tissue and the fibres in the stele area varied depending upon the thickness of the root. Presence of lateral root hairs and hardy nature of root made it a difficult system to be dealt with while carrying out microtomy.

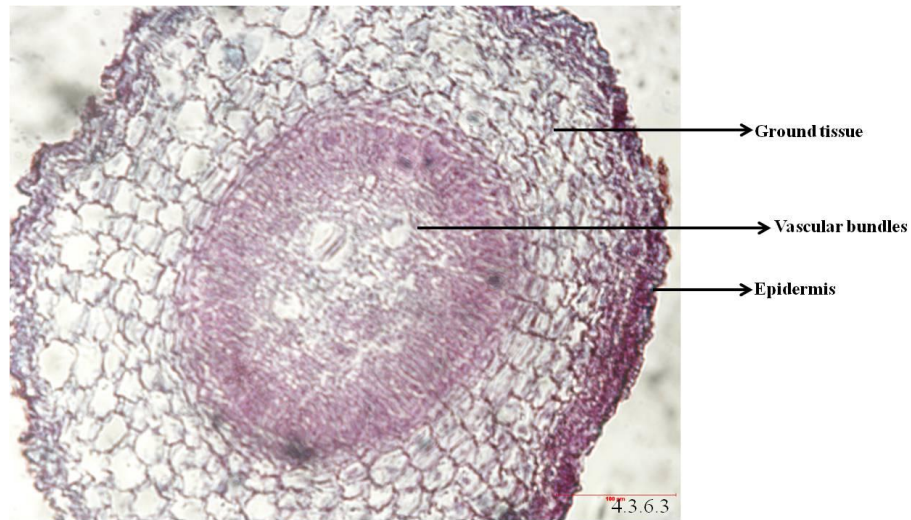
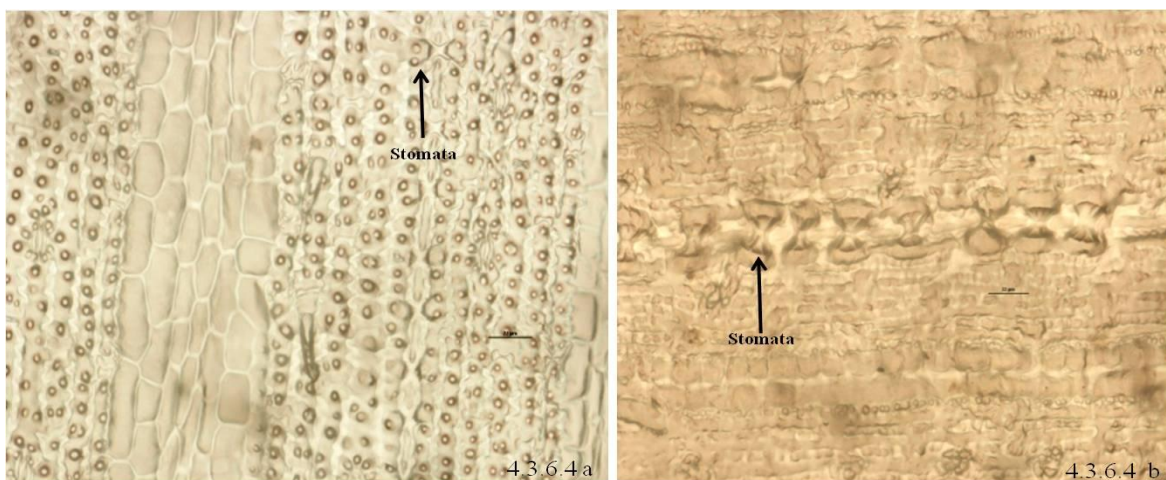


Figure 4.3.6.3. T.S of root showing internal structure (100 X).

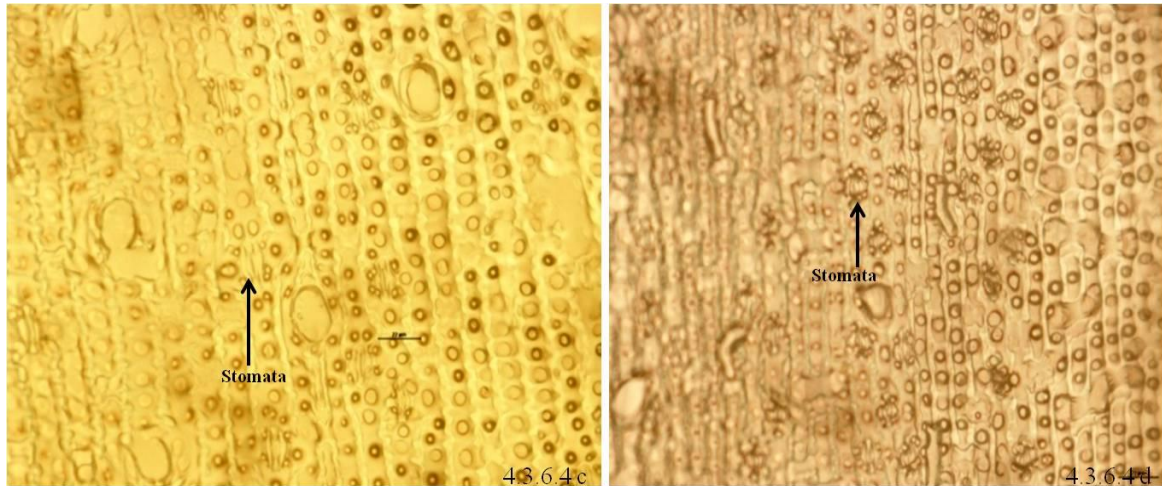
An evaluation of comparative anatomical studies carried out in the *in vitro* and *ex vitro* plants did not show any major difference in their internal structure except for the more lignified tissue present in the field plants.

4.3.6.4 Stomatal Studies (Light Microscopy)

Stomatal number and stomatal index were found to be more in the *ex vitro* raised plants when compared to the *in vitro* grown plants. *In vitro* grown plant had a stomatal density of 20.0 ± 1.0 in $68 \mu\text{m}^2$ on the adaxial surface (Figure 4.3.6.4 a) as compared to the *ex vitro* grown leaf showing a stomatal density of 25.0 ± 2.0 (Figure 4.3.6.4 b). Similarly, stomata on abaxial surface of *in vitro* grown plant had a density of 29.66 ± 1.52 (Figure 4.3.6.4 c) as compared to 37.0 ± 2.0 (Figure 4.3.6.4 d) of *ex vitro* grown plant. It was also observed that more number of stomata were open on abaxial as compared to the adaxial side. Shape of stomata observed was typical dumb-bell shaped. Silica bodies were invariably present.



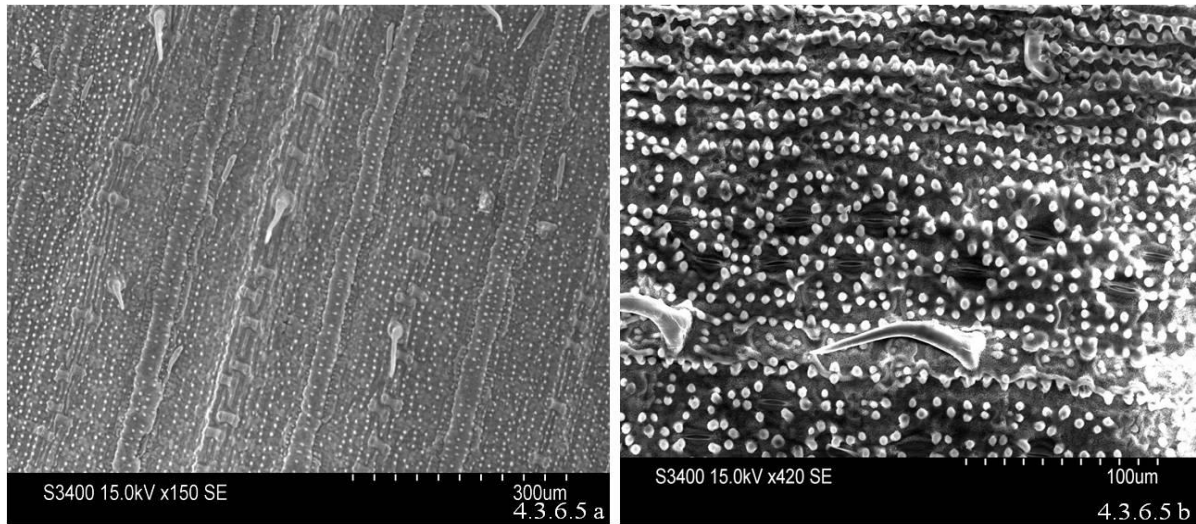
Figures 4.3.6.4 a, b. Stomata on adaxial surface of leaves of *in vitro* and *ex vitro* grown plants.



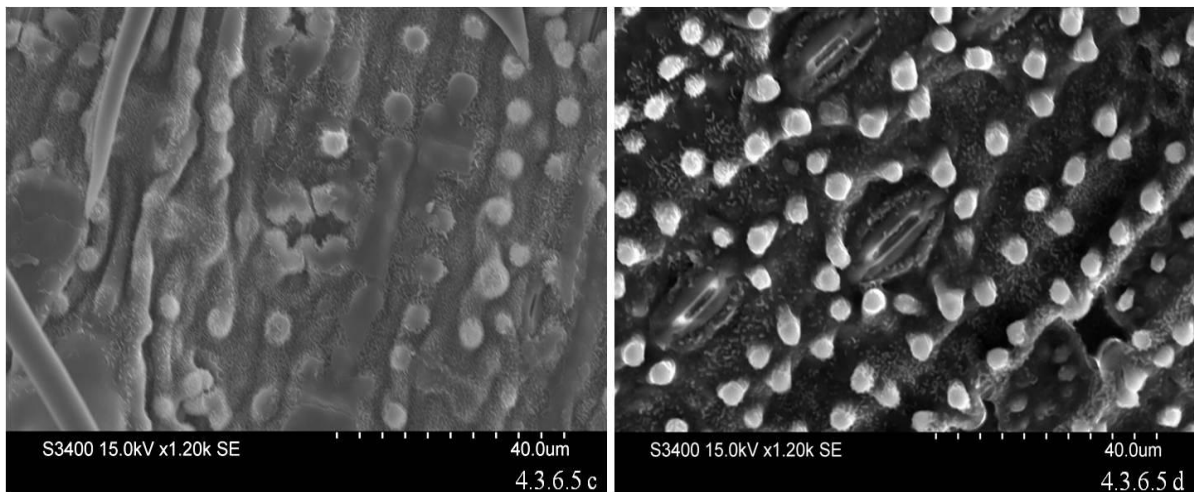
Figures 4.3.6.4 c, d. Stomata on abaxial surface of leaves of *in vitro* and *ex vitro* grown plants.

4.3.6.5 Stomatal Studies (Scanning Electron Microscopy)

Scanning Electron Microscope (SEM) studies were conducted to compare the stomatal density of the *in vitro* and *ex vitro* grown plants. Further the comparison was also done between the adaxial and the abaxial surfaces of the leaves. The electron micrograph pictures of the adaxial surface of *in vitro* grown leaves depicted a stomatal density of 37.0 ± 2.0 in 676 mm^2 (Figure 4.3.6.5 a) as compared to the stomatal density of 78.0 ± 6.2 in 676 mm^2 (Figure 4.3.6.5 b) in *ex vitro* grown leaf. Stomata were typical dumb bell shaped and silica bodies were seen scattered here and there in the whole leaf lamina. Further, it was found that abaxial surface of *in vitro* grown leaf had a stomatal density of 43.3 ± 3.70 in 105.03 mm^2 (Figure 4.3.6.5 c) as compared to *ex vitro* grown leaf showing stomatal density of 63.00 ± 1.5 in 105.03 mm^2 (Figure 4.3.6.5 d). Hence, the total number of stomata per leaf was found to be more in *ex vitro* plants (in same area) which is due to enormous leaf area growth after transfer to *ex vitro* conditions. More number of stomata were open in the *in vitro* grown plants as compared to hardened plants. The reason was that the stomata have not started functioning as yet in the *in vitro* plants as compared to functional stomatal apparatus in the acclimatized plants. Microhairs like protective structures were seen more conspicuously in the acclimatized plants mainly on the abaxial surface in costal and intercostal regions of the leaves.



Figures 4.3.6.5 a, b Scanning electron microscopy (SEM) images showing stomata on the adaxial surface of *in vitro* and *ex vitro* grown leaf respectively.



Figures 4.3.6.5 c, d. Scanning electron microscopy (SEM) images showing stomata on the abaxial surface of *in vitro* grown leaf and *ex vitro* grown leaf respectively.

4.3.6.6 Leaf Area Index (LAI)

Leaf area index (LAI) of *in vitro* grown plants was less i.e. 1.10 ± 0.46 as compared to 4.78 ± 0.63 of the hardened plants as also shown in [Table 4.3.6.6](#). There was an increase in CO₂ concentration and light intensity in green house as compared to the *in vitro* conditions, thus resulting in higher rate of metabolism in the acclimatized plants accounting for an increased LAI.

4.3.6.7 Relative water content (RWC) and Water Saturation Deficit (WSD)

RWC is closely related with cell volume and reflects the balance between water supply to the leaf and transpiration rate. This influences the ability of plant to recover from water stress. *In vitro* grown plants were found to be better in terms of RWC showing a value of 62.3 ± 1.5 % and consequently less WSD of 37.7 ± 1.5 % whereas *ex vitro* plants depicted low values of RWC nearly 50.5 ± 3.0 % as shown in [Table 4.3.6.6](#).

4.3.6.8 Electrolyte Leakage

Electrolyte leakage is the predictive measure of the membrane permeability. An estimation of cell damage and hardness was made by comparing the conductivity of leaked contents from injured and uninjured tissues in water. In the present study, *in vitro* grown plants proved to be better in terms of less electrolyte leakage of 58.2 ± 4.6 % as compared to *ex vitro* plants showing a value 70.15 ± 2.5 (Table 4.3.6.6). Hence, loss of solutes was greater in the hardened plants.

4.3.6.9 Proline Content

Proline plays an important role in cellular homeostasis including redox balance and energy status. Proline helps in acclimatization of plants as it has the tendency to become uncharged at neutral pH and hence become a highly soluble solute in water. Proline content increases as water stress increases under *in vivo* conditions. It seems proline may play a role in minimizing the damage caused by dehydration as it acts as a compatible osmolyte. In the present study *ex vitro* acclimatized plants showed an increased proline content of 2.94 ± 0.23 $\mu\text{M/g}$ as compared to 2.27 ± 0.13 $\mu\text{M/g}$ of *in vitro* grown plants (Table 4.3.6.6).

4.3.6.10 Lignin Content

Lignin is a complex phenolic polymer important for mechanical support, water transport and defense. It also affects the digestibility and hence alters the edible properties of bamboo species. Considerable difference in the lignin content of *in vitro* and hardened plants was observed. Hardened plants displayed more of the lignin content 41.20 ± 3.1 mg/l as compared to the *in vitro* grown plants having a value of 33.13 ± 4.1 mg/l as depicted in Table 4.3.6.6. This was due to more growth in the culms.

4.3.6.11 Starch Estimation

Starch is a carbohydrate made up of long chains of glucose molecules attached to one another and is the main storage reserve of plants. Lower levels of starch (2.02 ± 0.11) mg g⁻¹ fr.wt. in the *ex vitro* grown plants can be attributed to their more vigorous growth in field conditions as compared to slow growth of *in vitro* grown plants depicting a value of 2.33 ± 0.10 mg g⁻¹ fr.wt. as shown in Table 4.3.6.6. Utilization of starch for growth and metabolism has been the reason for its decreased level.

4.3.6.12 Total Soluble Sugars (TSS)

Due to the increased irradiance and gaseous exchange, there is an increase in the photosynthetic activity in *ex vitro* grown plants resulting in corresponding decrease in total soluble sugars (TSS) levels viz. 12.00 ± 2.2 mg g⁻¹ fr.wt. as compared to a value of $16.10 \pm$

1.0 in the *in vitro* plants as shown in Table 4.3.6.6. This is due to more consumption of carbohydrates by the actively growing plants accounting for their increase in size and overall growth.

4.3.6.13 Chlorophyll Content by Ultrasonics

Survival of plants to the *ex vitro* conditions depends upon their ability to carry out photosynthesis which in turn is enhanced by the amount of chlorophyll in the leaves. *In vitro* and *ex vitro* grown culms were peeled and mixed in various solvents for comparison of the chlorophyll contents using the technique of ultrasonics. Acetone, DMSO and DMF were the solvents tested in the present investigation. On using any of the three as a solvent, chlorophyll was found to be more in the *ex vitro* grown plants as compared to the *in vitro* plants. Chlorophyll in acetone showed the best stability followed by DMSO and DMF. In acetone, DMSO and DMF extracts, the total chlorophyll contents were 22.21 ± 2.1 , 14.64 ± 1.71 and 7.91 ± 3.10 mg per gm of epidermis meal respectively as depicted in Table 4.3.6.13. Further the amount of chlorophyll a was found to be more as compared to chlorophyll b.

Table 4.3.6.6 Effect of *in vitro* and *ex vitro* environment on biochemical and physiological parameters in *B. bambos*

Parameters	<i>In vitro</i>	<i>Ex vitro</i>	Remarks
Relative Water Content%	62.3 ^a ± 1.5	50.5 ^b ± 3.0	Plant water status in terms of cellular hydration
Water saturation Deficit	37.7 ^a ± 1.5	49.5 ^b ± 3.0	Plant water balance
Electrolyte Leakage %	58.2 ^b ± 4.6	70.1 ^a ± 2.5	Cell membrane stability
Leaf Area Index	1.1 ^b ± 0.4	4.7 ^a ± 0.6	More growth and vigor of <i>ex vitro</i> plants
Lignin Content mg/l	33.1 ^b ± 4.1	41.2 ^a ± 3.1	Digestion is inversely co-related with lignin which is a source of fiber in food
TSS mg g ⁻¹ fr.wt.	16.1 ^a ± 1.0	12.0 ^b ± 2.2	TSS indicated utilization of sugars for growth and metabolism
Starch Content mg g ⁻¹ fr.wt.	2.3 ^a ± 0.1	2.02 ^b ± 0.1	Reserve pool of carbon
Proline Content μM/g	2.2 ^b ± 0.1	2.9 ^a ± 0.2	Signaling molecule and cellular homeostasis

Table 4.3.6.13 Chlorophyll content in culms of *in vitro* and *ex vitro* grown plants by ultrasonics

Solvents	<i>In vitro</i> (mg g ⁻¹ fr.wt.)	<i>Ex vitro</i> (mg g ⁻¹ fr.wt.)	Remarks
Acetone	12.03 ^a ± 1.33	22.21 ^a ± 2.1	More stable and less abrasive solvent
DMSO	8.03 ^b ± 1.16	14.64 ^b ± 1.71	Less toxic than DMF
DMF	6.70 ^c ± 2.69	7.91 ^b ± 3.10	More viscous, toxic and less stable

Values are mean ± SD. ^{a,b} are mean separation within columns by Duncan's multiple range test, P ≤ 0.05

Objective 2: Checking their clonal fidelity by RAPD techniques to ensure quality and to study the field performance of the *in vitro* raised plants.

4.4 Molecular Characterization

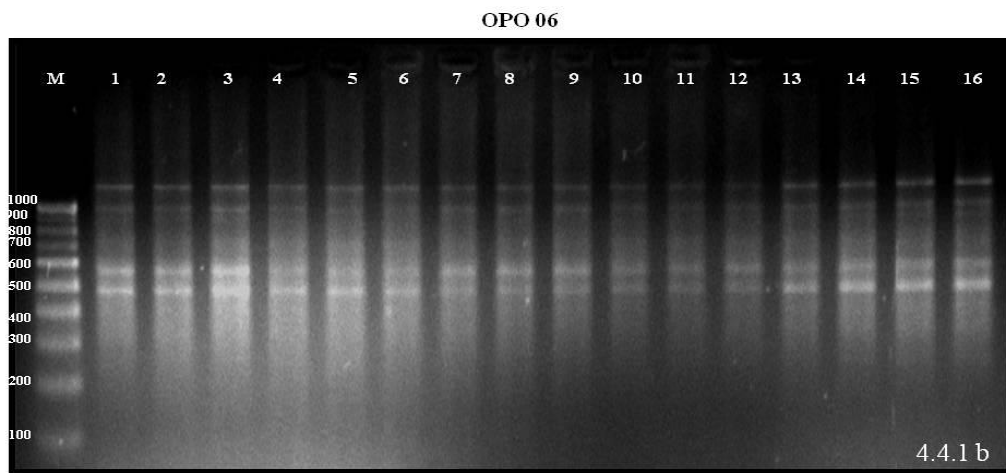
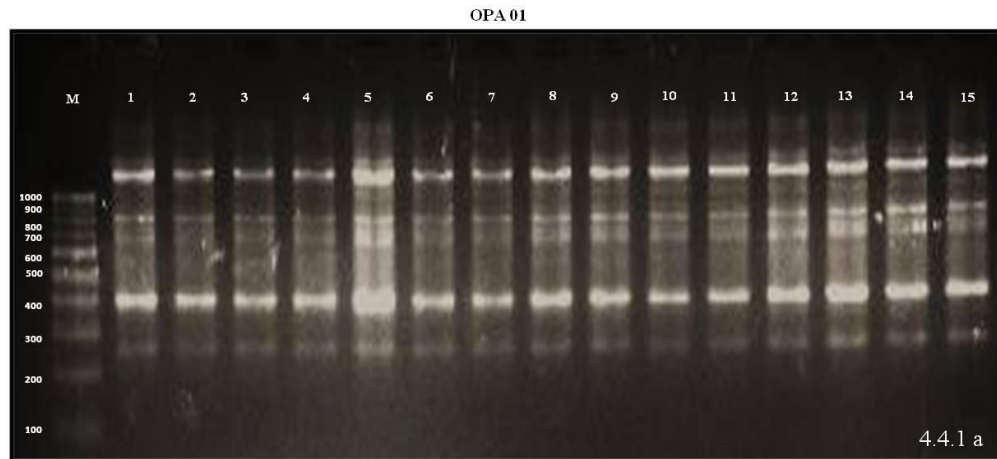
4.4.1 Clonal Fidelity of *In Vitro* Raised *Dendrocalamus membranaceus* Plants

Polymerase chain reaction (PCR) based techniques such as RAPD and ISSR were immensely useful in establishing the genetic stability of *in vitro* regenerated plants in the current investigation. These two types of molecular markers were used to test the clonal fidelity due to their simplicity and cost effectiveness. Total genomic DNA of the mother plant and the *in vitro* raised clones was extracted from young leaf tissue by using cetyl trimethyl ammonium bromide (CTAB) method. For ascertaining the clonal fidelity, 15 randomly selected plants, following atleast 6 months of hardening, were subjected to RAPD and ISSR analyses. Out of 35 scanned RAPD markers, only 30 primers were informative as they produced 73 amplified products (Table 4.4.1 a). During ISSR analysis of 20 markers, only 15 ISSR primers gave 38 scorable bands (Table 4.4.1 b). Overall a total of 45 scanned markers, 111 amplified products were obtained. The size of fragments varied in the range of 200 bp to more than 1 kb. Optimum T_m for RAPD markers falls near 37 °C and that for ISSR markers the range falls between 45.5 to 54 °C. For RAPD analysis, OPA series gave best amplification followed by OPO and OPT series (Figures 4.4.1 a and b). For ISSR markers, the best amplification was obtained with UBC series with UBC 834 giving 5 scorable bands followed by UBC 840 which gave 4 and UBC 844, UBC 850, UBC 857 and UBC 888 which giving 3 scorable bands each as shown in Figures 4.4.1 c and d. All banding profiles from the micropropagated plants were monomorphic and similar to those of mother plant thereby, confirming true to type nature of *in vitro* raised plants.

Table 4.4.1 a. Random Amplified Polymorphic DNA (RAPD) primers used to verify *D. membranaceus* clones

Primers	5'-3' motif	Scorable bands	Monomorphic bands	Polymorphic bands	Range of amplification (bp)
OPA 01	CAG GCC CTT C	7	7	0	250,400,700,750,800,1100,1150
OPA 02	TGC CGA GCT G	3	3	0	400,600,800
OPA 03	AGT CAG CCA C	2	2	0	350,700
OPA 04	AAT CGG GCT G	5	5	0	350,600,700,800,1100
OPA 05	AGG GGT CTT G	5	5	0	250,350,500,600,700
OPA 08	GTG ACG TAG G	5	5	0	250,500,600,700,800
OPA 09	GGG TAA CGC C	6	6	0	300,400,450,500,550,900
OPA 11	CAA TCG CCG T	1	1	0	500
OPA 12	TCG GCG ATA G	1	1	0	1000
OPA 15	TTC CGA ACC C	5	5	0	350,400,450,500,600
OPA 17	GAC CGC TTG T	2	2	0	500,1000
OPA 18	AGG TGA CCG T	4	4	0	500,600,1000,1100
OPO 02	ACG TAG CGT C	3	3	0	600,800,1300
OPO 04	AAG TCC GCT C	1	1	0	1000
OPO 06	CCA CGG GAA G	4	4	0	500,600,1000,1100
OPO 10	TCA GAG CGC C	1	1	0	350
OPO 12	CAG TGC TGT G	1	1	0	1100
OPO 13	GTC AGA GTC C	1	1	0	1150
OPO 16	TCG GCG GTT C	1	1	0	500
OPO 18	CTC GCT ATC C	1	1	0	1100
OPT 02	GGA GAG ACT C	3	3	0	550,700,900
OPT 04	CAC AGA GGG A	1	1	0	400
OPT 05	GGG TTT GGC A	1	1	0	500
OPT 06	CAA GGG CAG A	1	1	0	500
OPT 07	GGC AGG CTG T	1	1	0	350
OPT 10	CCT TCG GAA G	1	1	0	1200
OPT 11	TTC CCC GCG A	1	1	0	750
OPT 15	GGA TGC CAC T	1	1	0	1000
OPT 17	CCA ACG TCG T	1	1	0	400
OPT 19	GTC CGT ATG G	3	3	0	100,1100,1500
Total	30	73	73	0	100-1500

OP series sequences of Operon Technologies-Alameda, USA

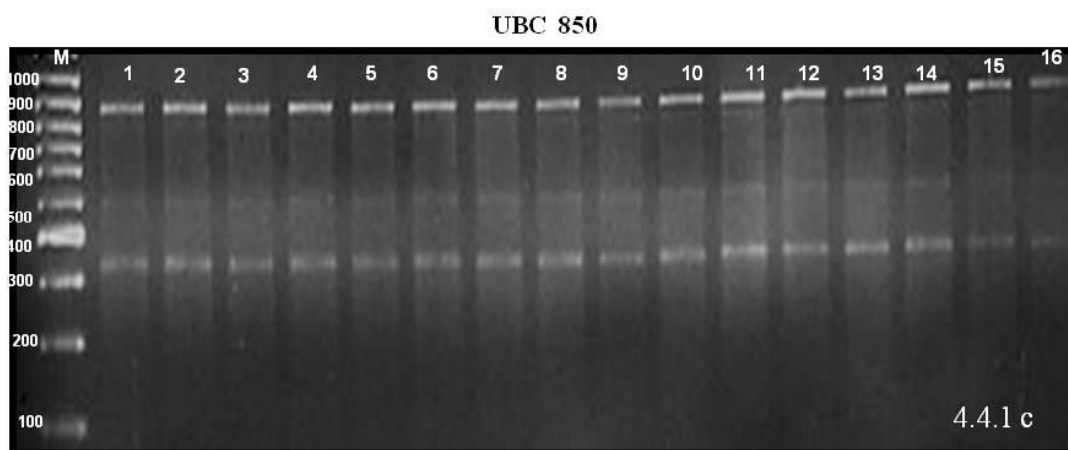


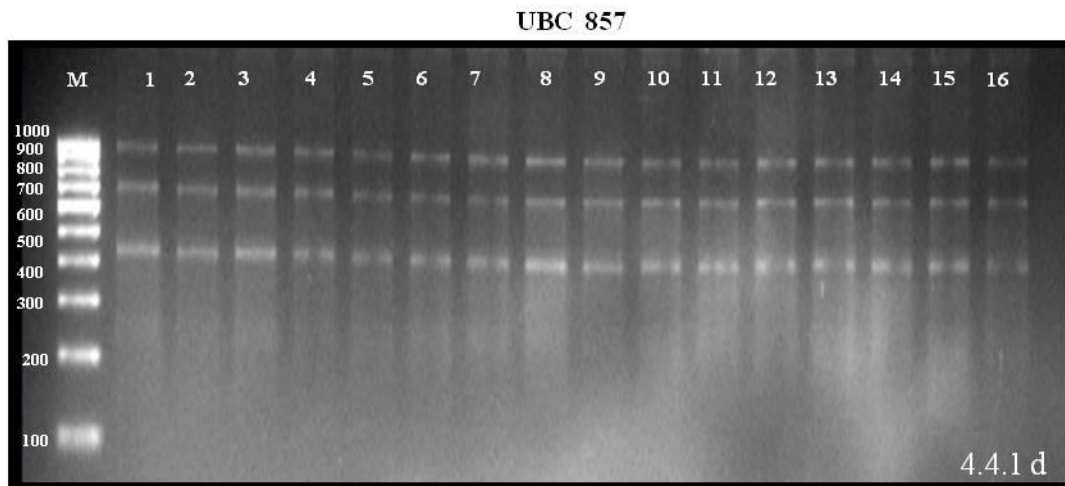
Figures 4.4.1 a, b. Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA primers with OPA 01 series and OPO 06 series. M represent 100 bp ladder, 1 is mother or control plant. 2-16 are *in vitro* raised clones of *D. membranaceus*.

Table 4.4.1 b The Inter Simple Sequence Repeats (ISSR) Markers Utilized to Verify *D. membranaceus* Clones

Primer	5'-3' motif	T _m °C	T _a °C	Scorable Bands	Monomorphic bands	Polymorphic bands	Range of Amplification (bp)
UBC 807	(AG) ₈ T	47.0	44.5	1	1	0	400
UBC 808	(AG) ₈ C	48.8	45.5	1	1	0	700
UBC 810	(GA) ₉ T	45.4	43.5	3	3	0	600, 700, 800
UBC 811	(GA) ₈ C	46.8	44.5	3	3	0	300, 500, 700
UBC 812	(GA) ₈ A	45.7	43.0	1	1	0	600
UBC 818	(CAC ACA) ₂ CAC AG	51.0	48.0	2	2	0	450, 800
UBC 830	(TG) ₈ G	52.0	50.0	3	3	0	400, 700, 850
UBC 834	(AG) ₈ YT	49.2	47.0	5	5	0	400, 450, 600, 700, 800
UBC 835	(AG) ₈ C	48.8	44.0	2	2	0	800, 900
UBC 836	(AG) ₈ YA	48.9	45.5	1	1	0	850
UBC 840	(GA) ₈ YT	47.4	42.0	4	4	0	500, 600, 1000, 1100
UBC 844	(CT) ₈ RC	48.6	44.5	3	3	0	300, 500, 600
UBC 850	(GT) ₈ YC	52.7	49.5	3	3	0	450, 600, 1000
UBC 857	(AC) ₈ YG	54.3	50.0	3	3	0	450, 700, 900
UBC 888	BDBC(AC) ₅ A	47.3	44.4	3	3	0	450, 500, 700
Total	15	-	-	38	38	0	300-1100

UBC series sequences of University of British Columbia, Canada
 B = (C, G, T i.e. not A); D = (A, G, T i.e. not C); R = (A, G); Y = (C, T)





Figures 4.4.1 c, d ISSR products generated from 15 *in vitro* regenerated plants and mother plant of *D. membranaceus* amplified with primers UBC 850 and UBC 857 showing monomorphic pattern. Lane M represent 100 bp marker, 1 is mother or control plant. 2-16 represent *in vitro* raised clones of *D. membranaceus*.

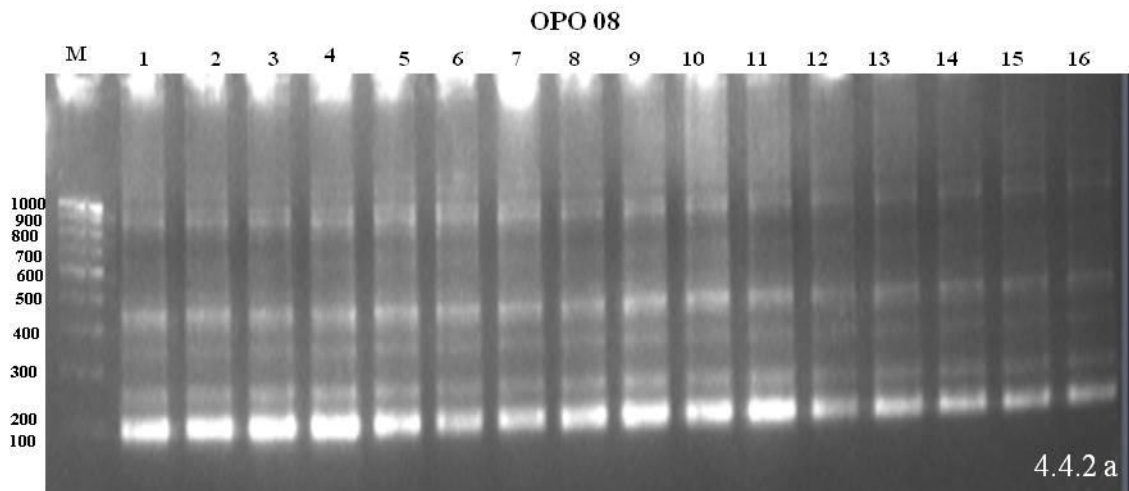
4.4.2 Clonal Fidelity of *In Vitro* Raised *Bambusa balcooa* Plants

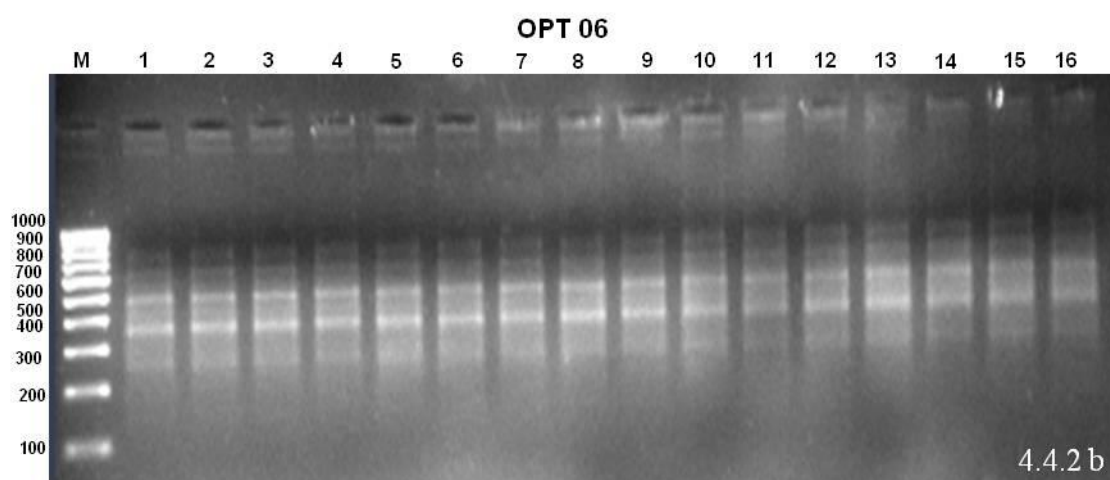
For ascertaining the clonal fidelity of tissue culture raised plants following at-least 6 months of their hardening in green house, 15 randomly selected plants and the mother plant were subjected to RAPD and ISSR analyses. Our results depicted that not all primer combinations produce either reliable or sufficient data. Hence, preliminary screening of primers was performed to eliminate the primers giving insufficient data. Out of 25 RAPD markers scanned, only 21 primers produced 61 amplicons (Table 4.4.2 a). In case of ISSR analysis using 15 markers only, 10 ISSR primers gave 28 scorable bands (Table 4.4.2 b). A total of 31 scanned markers, 89 amplified products were obtained. Consistent well resolved fragments in the size range of 100 to 1500 kb were manually scored. Optimum T_m for RAPD markers falls near 37 °C and that for ISSR markers, the range falls between 41.5 to 50.5 °C. For RAPD analysis, OPO series gave the best amplification followed by OPA and OPT series. Amplifications of OPO 08 and OPT 06 are shown in Figures 4.4.2 a and b. For ISSR, markers UBC 810, 811, 888 gave maximum amplified products in the range of 250-1500 bp. ISSR products amplified with primer UBC 810 and UBC 850 are shown in Figures 4.4.2 c and d. In our study, no variation was reported among the tissue culture raised progeny and the mother plant in the banding profiles generated by RAPD and ISSR markers. Hence, clonal fidelity was established with no apparent genetic variations.

Table 4.4.2 a Random Amplified Polymorphic DNA (RAPD) Primers Used to Verify *B. balcooa* Clones

Primers	5'-3' motif	Scorable Bands	Monomorphic Bands	Polymorphic Bands	Range of Amplification (bp)
OPA 01	CAG GCC CTT C	4	4	0	550, 600, 700, 800
OPA 02	TGC CGA GCT G	2	2	0	300, 500
OPA 04	AAT CGG GCT G	3	3	0	450, 550, 700
OPA 12	TCG GCG ATA G	4	4	0	250, 350, 500, 600
OPA 17	GAC CGC TTG T	4	4	0	400, 900, 1000, 1200
OPA 19	CAA ACG TCG G	3	3	0	700, 800, 1100
OPO 02	ACG TAG CGT C	2	2	0	300, 500
OPO 05	CCC AGT CACT	4	4	0	200, 300, 700, 900
OPO 07	CAG CAC TGA C	2	2	0	600, 1000
OPO 08	CCT CCA GTG T	6	6	0	100, 150, 250, 350, 800, 900
OPO 14	AGC ATG GTC C	1	1	0	1200
OPO 15	TGG CGT CCT T	5	5	0	200, 250, 500, 600, 1000
OPO 16	TCG GCG GTT C	2	2	0	500, 1100
OPO 18	CTC GCT ATC C	1	1	0	500
OPT 06	CAA GGG CAG A	3	3	0	350, 700, 800
OPT 09	CAC CCC TGA G	1	1	0	500
OPT 10	CCT TCG GAA G	1	1	0	300
OPT 12	GGG TGT GTA G	4	4	0	400, 450, 500
OPT 13	AGG ACT GCC A	2	2	0	100, 250
OPT 16	GGT GAA CGC T	4	4	0	500, 600, 900, 1100
OPT 18	GAT GCC AGA C	3	3	0	400, 700, 1200
Total	21	61	61	0	100-1200

OP series sequences of Operon Technologies-Alameda, USA



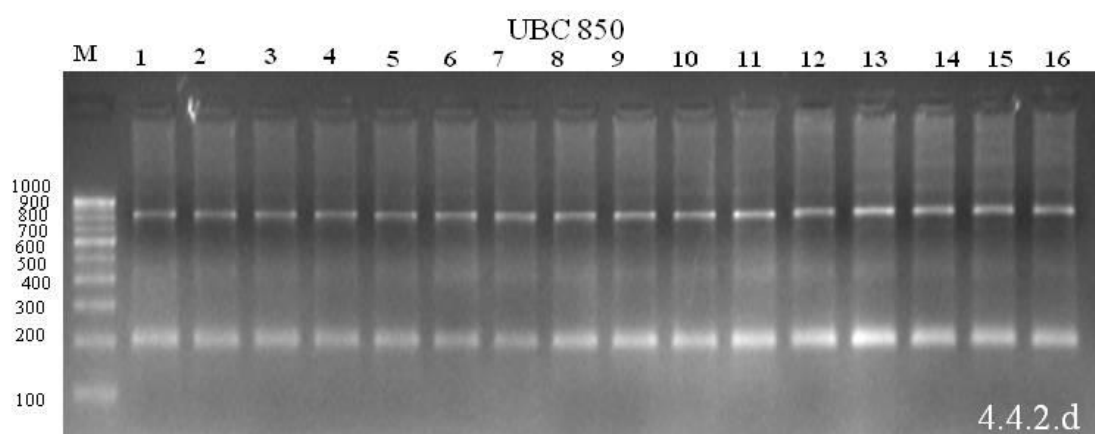
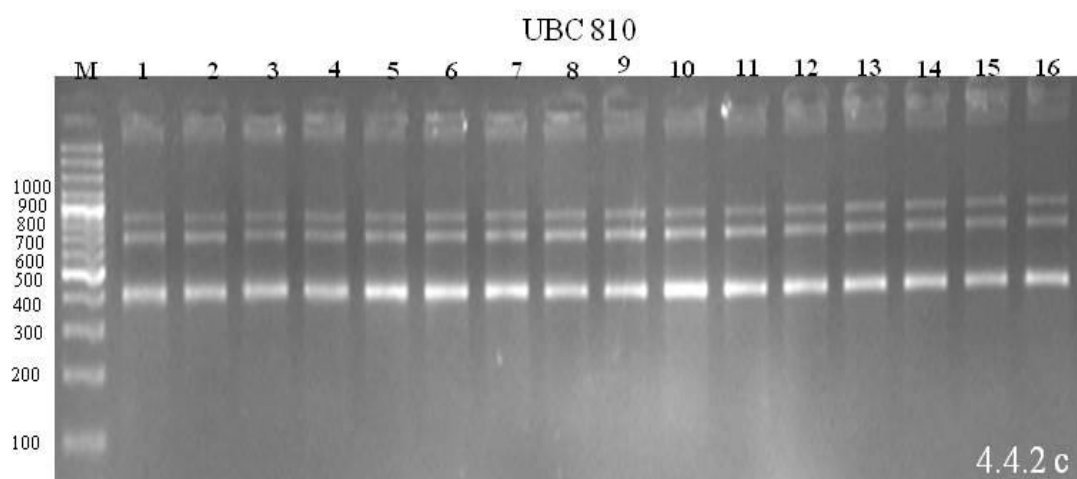


Figures 4.4.2 a, b Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA (RAPD) primers OPO 08 and OPT 06. Lane M represents 100-bp ladder, Lane 1 represents mother plant, Lane 2-16 represent *in vitro* raised clones of *B. balcooa*.

Table 4.4.2 b The Inter Simple Sequence Repeats (ISSR) Markers Utilized to Verify *B. balcooa* Clones

Primer	5'-3' motif	T _m °C	T _a °C	Scorable Bands	Monomorphic Bands	Polymorphic Bands	Range of Amplification (bp)
UBC 810	(GA) ₉ T	45.4	42.5	4	4	0	400, 700, 900, 1500
UBC 811	(GA) ₈ C	46.8	44.0	4	4	0	250, 500, 600, 1400
UBC 812	(GA) ₈ A	45.7	41.5	3	3	0	400, 500, 800
UBC 815	(CT) ₈ G	46.8	44.0	2	2	0	900, 1100
UBC 818	(CAC ACA) ₂ CAC AG	51.0	48.5	3	3	0	500, 700, 1100
UBC 834	(AG) ₈ YT	49.2	46.0	3	3	0	150, 300, 350
UBC 844	(CT) ₈ RC	48.6	45.0	1	1	0	400
UBC 850	(GT) ₈ YC	52.7	50.0	3	3	0	200, 400, 900
UBC 857	(AC) ₈ YG	54.3	50.5	1	1	0	100
UBC 888	BDBC(AC) ₅ A	47.3	43.5	4	4	0	300, 400, 600, 1400
Total	10	–	–	28	28	0	100-1500

UBC series sequences of University of British Columbia, Canada
 B = (C, G, T i.e. not A); D = (A, G, T i.e. not C); R = (A, G); Y = (C, T)



Figures 4.4.2 c, d. ISSR products generated from 15 *in vitro* regenerated plants and mother plant of *B. balcooa* amplified with primers UBC 810 and UBC 850 showing monomorphic pattern. Lane M represents 100-bp ladder, Lane 1 represents mother plant, Lane 2-16 represent *in vitro* raised clones of *B. balcooa*

4.4.3 Checking the Clonal Fidelity of *In Vitro* Raised *Bambusa bambos* Plants

For ascertaining the clonal fidelity, 15 randomly selected plants were subjected to RAPD and ISSR analysis. PCR fragments were separated on 1.8% agarose gel. The PCR reactions were mixed with equal volume of loading buffer (98% glycerol containing 0.8 mM EDTA and 0.025% of each bromophenol blue and xylene cynol) maintained at -20 °C. Samples were loaded in preheated PCR which run at 60 Volt for 1.5 up to 2.0 hrs depending upon the fragment size to be separated. Out of 15 RAPD markers scanned, only 10 primers produced amplicons (Table 4.4.3 a). In case of ISSR analyses using 10 markers only, 5 markers gave scorable bands (Table 4.4.3 b). Out of total 25 scanned markers, amplified products were obtained in the size range of 100 to 1500 bp. Optimum T_m for RAPD markers falls near 37 °

C and that for ISSR markers, the range falls between 45.4 to 54.3 °C. For RAPD analysis, OPA series gave the best amplification (Figure 4.4.3 a). For ISSR analysis, UBC 818 gave the maximum amplified products in the range of 200 to 1400 bp (Figure 4.4.3 b). UBC 810 gave four bands but two of the bands were faint and were not sharp. In the present study, no variation was reported among *in vitro* raised progeny and the mother plant in the banding profiles generated by RAPD and ISSR markers. Hence, molecular analysis confirmed that these plants were genetically similar and can be used as elite plants.

Table 4.4.3 a Random Amplified Polymorphic DNA (RAPD) Primers Used to Verify *B. bambos* Clones

Primers	5'-3' motif	No of scorable bands	Monomorphic bands	Polymorphic bands	Range of amplification (bp)
OPA 01	CAG GCC CTT C	3	3	0	1000,1100,1300
OPA 02	TGC CGA GCT C	3	3	0	400,900,1000
OPA 19	CAA ACG TCG G	3	3	0	450,700,1000
OPT 10	CCT TCG GAA G	3	3	0	300,500,1500
OPT 18	GAT GCC AGA C	2	2	0	800,900
OPO 06	CCA CGG GAA G	1	1	0	1500
OPO 07	CAG CAC TGA C	2	2	0	450,1400
OPO 08	CCT CCA GTG T	2	2	0	200,1300
OPO 15	TGG CGT CCT T	2	2	0	800,1500
OPO 18	CTC GCT ATC C	2	2	0	200,1300
Total	10	23	23	0	200-1500

OP series sequences of Operon Technologies-Alameda, USA

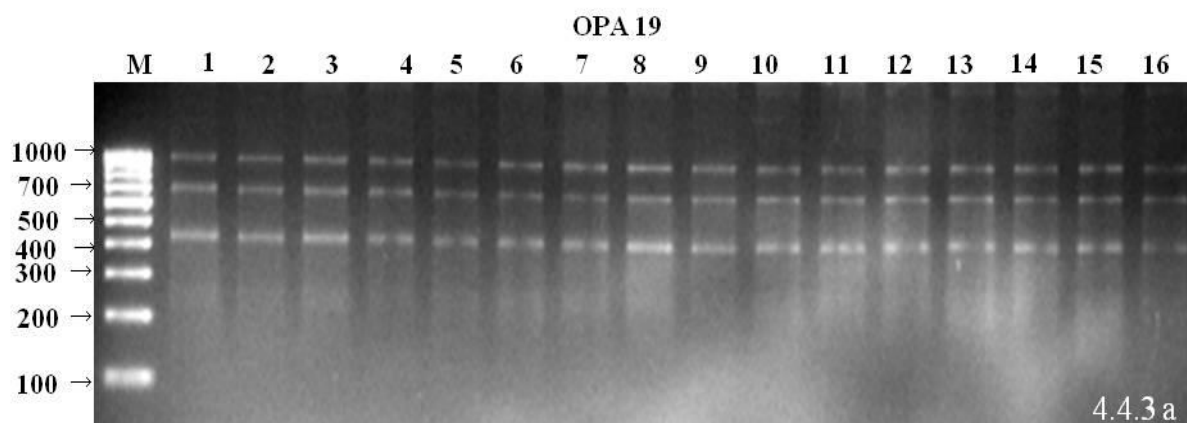


Figure 4.4.3 a. Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA (RAPD) primer OPA 19. Lane M represents 100-bp ladder, Lane 1 represents mother plant, Lane 2-16 represent *in vitro* raised clones of *B. bambos*.

Table 4.4.3 b The Inter Simple Sequence repeats (ISSR) Markers Utilized to Verify *B. bambos* clones

Primers	5'-3' motif	T _m °C	T _a °C	Scorable Bands	Monomorphic Bands	Polymorphic Bands	Range of Amplification (bp)
UBC 810	(GA) ₉ T	45.4	42.5	4	4	0	100,400,450,1000
UBC 812	(GA) ₈ A	45.7	41.5	2	2	0	100,350
UBC 818	(CACACA) ₂ CACAG	51.0	48.5	3	3	0	200,400,1400
UBC 834	(AG) ₈ YT	49.2	46.0	3	3	0	350,400,700
UBC 857	(AC) ₈ YG	54.3	50.5	2	2	0	400,1500
Total	5	-	-	14	14	0	100-1500

UBC series, Sequences of University of British Columbia, Canada
Y= (C,T)

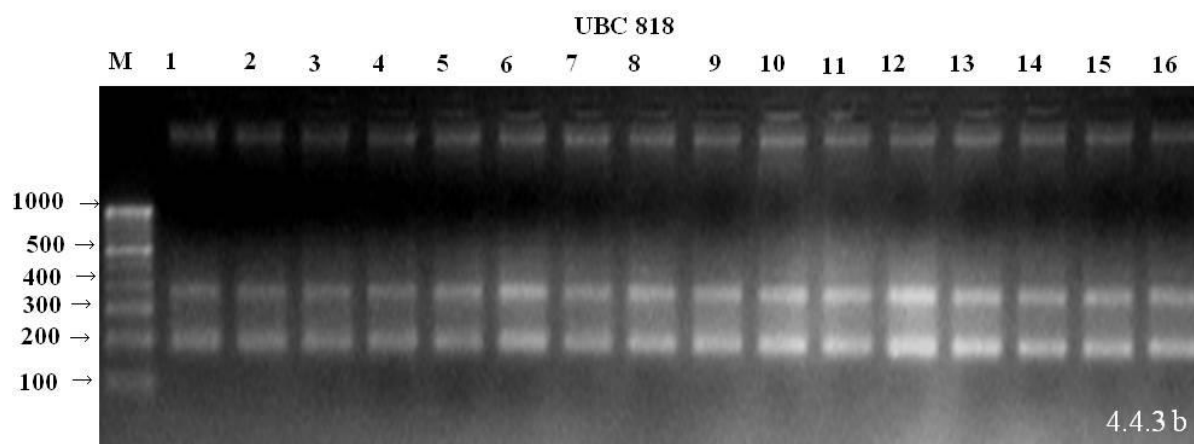


Figure 4.4.3 b. ISSR products generated from 15 *in vitro* regenerated plants and mother plants of *B. bambos* amplified with primer UBC 818 showing monomorphic pattern. Lane M represents 100-bp ladder, Lane 1 represents mother plant, Lane 2-16 represent *in vitro* raised clones of *B. bambos*.

4.5 Analysis of Field Performance of *In Vitro* Raised Plants

Field evaluation of tissue-culture raised plants and their performance (morphological and physiological) is important for long term assessment and commercial applications. The hardened plants of all the three species were initially acclimatized in high humid conditions of green house for a period of 5-6 months. The best time for their transfer to fields was beginning of rainy season (June-July) where they exhibited 100% survival. The plants were transplanted in the field to pits (2ft x 2ft x 2ft) at a plant to plant and row to row distance of 6m in a complete randomized block design (CRBD) as shown in [Table 4.5 a](#). Their field performance after 6 months was recorded by studying different plant parameters like height, number of shoots, number of leaves, number of axillary shoots per clump and number of internodes formed per shoot.

Under field conditions, *Dendrocalamus membranaceus* plants depicted maximum growth attaining height of 101.66 cm and on an average 294 leaves and 24.6 new shoots emerged during this period as shown in [Table 4.5 b](#)

In *Bambusa balcooa* the plants attained height upto 95.5 cm with corresponding increase in number of shoots and leaves ([Table 4.5 b](#)). Number of axillary shoot/clump and number of internodes/shoot also increased indicating their successful establishment under the field conditions. *In vitro* derived plants displayed normal development similar to the plants growing in the field.

In *Bambusa bambos*, hardened plants showed an increase in height upto 50 cm with corresponding increase in number of leaves (94) as shown in [Table 4.5 b](#). Increase in number of shoots to 12 with 7 axillary shoots per clump depicting overall growth and development of the plants and successful acclimatization under field conditions.

Table 4.5 a. Randomization of plants in field

Row 1	Row 2	Row 3
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5

Table 4.5 b. Data recorded after 6 months (hardened plants)

Parameter	<i>D. membranaceus</i>	<i>B. balcooa</i>	<i>B. bambos</i>	Inference
Height	101.66 cm	95.5 cm	50 cm	Increase in height indicated that plants acclimatized well after 6 months
No. of Shoots	24.6	20	12	Increase in number resulted in more Net Primary Productivity (NPP)
No. of Leaves	294	255	94	Increase in number and consequently increase in chlorophyll content in the <i>ex vitro</i> grown plants
No. of axillary shoots/clump	12.4	9.5	7	Increase in number indicating increase in biomass
No. of internodes/shoot	7.6	5.5	3	Increase in number resulting in overall growth and development of hardened plants

5. Discussion

Bamboos are among the economically most important plants worldwide and considered as an ideal renewable resource for biomass besides its innumerable applications in handicraft (local industries) to industrial scale paper mills. An estimate regarding the future use of bamboo indicates that there will be a huge shortage of bamboo planting material in long term and hence, intensive research on improved propagation system is required (Nadgauda, 1997 a). For mass scale propagation, classical techniques are largely insufficient and inefficient and are beset with many problems such as seed sterility, non availability of seeds, unpredictable flowering nature and bulkiness of rhizome (Arya et al. 2002). It is opined that after a stand has bloomed and died, the new seedlings are completely unprotected from cattle and fire (Tewari, 1988). A lot of natural bamboo forests have disappeared from their habitats due to all these prevailing factors.

In order to enhance production especially against a background of over-exploitation, a sharper focus especially on limited number of high priority species needs to be given. Among the 20 taxa of bamboo listed by IUCN, *Bambusa balcooa* and *B. bambos* are accorded high priority for international action. Further 18 taxa have been noted to be important which includes *Dendrocalamus membranaceus* among them (Bystriakova et al. 2004). Although, some protocols for micropropagation of these species from nodal explants are already available (Yasodha et al. 1997; Arya and Sharma, 1998; Das and Pal, 2005a; Islam and Rahman, 2005; Mudoi and Borathakur, 2009 and Negi and Saxena, 2011 a) many aspects of research are still needed including propagation management for more efficient mass propagation, improvements in rooting and acclimatization processes, biochemical and physiological parameters and modifying *in vitro* and *ex vitro* conditions according to the need of the species. These will prove beneficial for large scale propagation of these species.

5.1 Micropropagation

Under the scope of present investigation, micropropagation protocols for three bamboo species namely *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos* were standardized using different techniques of micropropagation.

5.1.1 Culture Initiation

For the initiation of cultures, judicious selection of the explant is fundamental because different explant tissue types within the same plant differ in their response to a particular culture condition and the season of the year. Nodal explants are mainly preferred due to their availability all the year round in sufficient numbers as compared to paucity and problems of

obtaining seeds. In the present study, among the various explants tested in all the three species, unexpanded axillary buds were found to be the best for raising the cultures as they responded favourably under culture conditions. The suitability of nodal segments having axillary buds has been reported in micropropagation of several bamboos like *Bambusa vulgaris* (Nadgir et al. 1984; Hirimburegama and Gamage, 1995 and Ramanayake et al. 2006), *B. balcooa* (Mudoj and Borathakur, 2009 and Negi and Saxena, 2011a); *B. glaucescens* (Banik, 1987); *B. bambos* (Arya et al. 1998 and Anand et al. 2013); *B. edulis* (Lin and Chang, 1998); *B. nutans* (Mehta et al. 2010); *Dendrocalamus hamiltonii* (Sood et al. 1992 and Agnihotri et al. 2009); *D. strictus* (Chaturvedi et al. 1993; Ravikumar et al. 1998 and Mukunthakumar et al. 1999); *D. longispathus* (Saxena and Bhojwani, 1993); *D. giganteus* (Ramanayake and Yakandawala, 1997 and Arya et al. 2006); *Thamnocalamus spathiflorus* (Bag et al. 2000); *Pseudoxyanthera stocksii* (Sanjaya et al. 2005); *Guadua angustifolia* (Jimenez et al. 2006 and Nadha et al. 2011) and many more. Leaf explants were employed for initiating cultures in *Phyllostachys bambusoides* (Komatsu et al. 2011) and *Bambusa glaucescens* (Jullien and Van, 1994) but in the present investigation, leaf explants did not show response on any of the media combinations tried and they turned achlorophyllous in due course of time. Seeds have also been used for initiation of cultures in *Dendrocalamus strictus* (Reddy et al. 2006) and *Ochlandra wightii* (Bejoy et al. 2012) as also used in present study in *Dendrocalamus membranaceus* (Brar et al. 2013). Till date, micropropagation protocols for many bamboo species have been successfully worked out but success with explants taken from mature plants of bamboos for propagation has been limited to a few cases only. Multiplication from adult culms is restricted by many factors and maturation of the tree species adversely affects the morphogenetic potential of the axillary buds (Pierik, 1990; Lin and Chang, 1998). Some of the probable factors responsible for poor success with adult explants include microbial contamination, browning of the medium and inconsistency in shoot multiplication as reported by Paranjothy et al. (1990).

5.1.2 Browning of the Medium

The browning phenomenon of cultured tissues has been attributed to oxidized phenolic compounds that frequently result in the early death of bamboo shoots in the *in vitro* as reported in *Bambusa oldhamii* (Huang et al. 2002) and *Phyllostachys nigra* (Ogita, 2005). The browning was shown to be pH dependent with standard pH 5.7 giving relatively low browning rates. Likewise, Das and Pal, (2005a) observed that the morphogenetic competence in *Bambusa balcooa* was adversely affected by the exudates released from the excised explants which caused browning of the medium and ultimately resulted in the necrotic

appearance of the shoots. They emphasized that the phenolic oxidation of *in vitro* cultures occurs when cells are ruptured during excision of the explant and the compartmentalized enzymes and substrates are released in the culture medium. To avoid browning, activated charcoal and/or polyvinylpyrrolidone (PVP) are often added to the tissue culture medium. In *Dendrocalamus strictus*, Saxena and Dhawan (1999) reported the addition of PVP to the medium in overcoming tissue browning in cultures. In *Phyllostachys nigra*, Ogita, (2005) demonstrated that when the brown necrotic portions of tissue were removed from the explant and frequent subculturing was performed, browning was reduced considerably. Similarly, in *Arundinaria callosa* (Devi and Sharma, 2009) and in *Gigantochloa artoviolaceae* (Bisht et al. 2010), quick transfer of the tissue to the fresh medium was recommended to reduce browning. In the present study, browning problem was overcome by frequent subculturing to the fresh medium.

5.1.3 Microbial Contamination

Initiation of cultures from mature tissue is one of the major problems encountered during micropropagation of bamboos and other woody perennials due to high contamination rates. The growth medium selected for *in vitro* propagation also serves as a good source of nutrients for microbial growth and these microbes further compete adversely with plants for nutrients (Oduyayo et al. 2007). The presence of microbes or latent infections in these plant cultures usually result in an increased culture mortality, variable growth, tissue necrosis and reduced shoot proliferation (Kane, 2003). Henceforth, various sterilization procedures are known to be adopted depending upon the explant used and the plant species. Use of ethanol, commercial bleach and mercuric chloride in raising aseptic cultures is quite a common practice (Huarte and Garcia, 2009; Liu et al. 2011). HgCl₂ has proved to be the best sterilant as it is very effective in controlling the pathogens as also discussed by Arya et al. (2008a) in *Dendrocalamus asper*. In the present investigation involving *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos*, a high dose of antimicrobial treatment including addition of antibiotics like streptomycin sulphate and tetracycline (0.02%) in addition to 15% sodium hypochlorite and 0.1% HgCl₂ was done to overcome the problem of high contamination rates. Similarly, in *D. membranaceus*, Yasodha et al. (1997) reported the use of kanamycin to overcome high contamination (60% bacterial and 7% fungal) during the initiation of cultures. The work of Kumari and Ramanayake, (1996) in *Bambusa vulgaris* and Ramanayake and Yakandawala, (1997) in *Dendrocalamus giganteus* reported an inverse relationship between *in vitro* bud break and culture contaminants. In *Dendrocalamus giganteus*, Ramanayake and Yakandawala, (1997) recommended the incorporation of benlate in the medium to

significantly reduce culture contaminants. Thakur and Sood, (2006) developed an advanced method of sterilization using stoppered glass measuring cylinder and found that the percentage of contamination was substantially reduced. Nadha et al. (2012) reported persistent appearance of bacterial contamination in the cultures of *Guadua angustifolia* which could be controlled by the addition of kanamycin and streptomycin sulphate in the medium. Contamination rate has been found to be directly influenced by the amount of rainfall at the time of explants collection. In the present study, highest rate of contamination was observed during the period of maximum rainfall especially in the month of July. Hence, rainfall had a direct influence on contamination rates and survival percentage of explants as also reported in *Bambusa balcooa* (Das and Pal, 2005a) and *Dendrocalamus asper* (Nadha et al. 2013).

5.1.4 Bud Break

McClure (1966) observed that bud break in bamboos varied with their position on the plant, the season of the year and the species. Bud break in *D. membranaceus* was found to be highest when mid-culm nodes of secondary branches were taken as explants. In *B. bambos* and *B. balcooa* single node cuttings from tertiary branches were observed to be the best explants. Likewise, in *Dendrocalamus giganteus*, the mid-culm nodes of secondary branches have been reported to give the best results for axillary shoot initiation (Ramanayake and Yakandawala, 1997). In tropical bamboos like *Bambusa vulgaris*, bud-break was found to be positively correlated with rainfall (Kumari and Ramanayake, 1996). According to Paranjothy et al. (1990), meristems in bamboos are most active only during rainy season and fresh cultures should be initiated during this period as also highlighted in *Dendrocalamus longispathus* (Saxena and Bhojwani, 1993); *D. giganteus* (Ramanayake and Yakandawala, 1997); *Bambusa balcooa* (Das and Pal, 2005a) and *B. nutans* (Yadav et al. 2008). But in our study involving *D. membranaceus* and *B. bambos*, spring season was found to be the best season for raising aseptic cultures with 90% and 93% bud break respectively. Rainy season (June to August) depicted 80% and 97% bud sprouting in these two plant species but was not a preferred season as it had a direct influence on contamination rate (95%) and survival percentage of explants.

5.1.5 Cytokinins and Axillary Shoot Proliferation

Presently, initial bud break in all the three species viz. *D. membranaceus*, *B. balcooa* and *B. bambos* occurred on basal MS medium without any growth regulators. However, only 2-3 shoots were formed and for further shoot proliferation, addition of BAP was necessary. The effectiveness of cytokinins especially BAP in promoting axillary shoot proliferation in bamboos is well documented as reported in *Dendrocalamus membranaceus* (Yasodha et al.

1997); *D. hamiltonii* (Agnihotri and Nandi, 2009); *D. giganteus* (Yasodha et al. 2010) *Melocanna bambusoides* (Devi and Sharma, 2009); *Gigantochloa atroviolaceae* (Bisht et al. 2010); *Bambusa balcooa* (Negi and Saxena, 2011a) and *B. bambos* (Anand et al. 2013). The superiority of BAP for shoot induction and multiplication may be due to the ability of plant tissues to metabolize BAP more readily or the ability of BAP to induce production of natural hormone such as zeatin within the tissue (Zaerr and Mapes, 1982 and Mukunthakumar et al. 1999). It has been observed in the present investigation that the use of higher concentration of BAP (13.2 μM) not only lowered the multiplication rate but also resulted in stunted shoots as also demonstrated in *B. nutans* and *D. membranaceus* (Yasodha et al. 1997). A high level of cytokinins is known to induce programmed cell death in cell cultures and yellowing of leaves in intact plant (Carimi et al. 2003 and Islam and Rahman, 2005). Our results are in consonance with the reports in *B. bambos* (Arya and Sharma, 1998); *B. nana* (Shirin and Arya, 2003); *B. wamin* (Arshad et al. 2005); *B. glaucescens* (Shirin and Rana, 2007) and *B. balcooa* (Negi and Saxena, 2011a) where higher concentration of BAP inhibited the shoot proliferation. Addition of Kn alone did not promote shoot proliferation and resulted in very less number of shoots (2-3) in all the three species under consideration. The shoots remained dormant and ultimately died. In our study on *B. bambos* and *D. membranaceus*, addition of Kn (1.16 μM) to BAP (4.4 μM) supplemented medium considerably enhanced the multiplication rate showing their synergistic effect. The synergistic effect of BAP and Kn resulting in enhanced rate of multiplication has been observed in many bamboo species like *D. strictus*, *B. arundinacea* and *B. vulgaris* (Nadgir et al. 1984; Shirin and Arya, 2003); *B. tulda* (Saxena, 1990); *B. wamin* (Arshad et al. 2005); *B. balcooa* (Negi and Saxena, 2011a) *D. giganteus* (Ramanayake and Yakandawala, 1997); *D. strictus* (Ravikumar et al. 1998); *Melocanna baccifera* (Kant et al. 2009) etc.

Presently in *B. balcooa*, MS medium containing BA as a lone growth regulator was quite effective in inducing multiple shoot proliferation forming 16.8 ± 1.1 shoots from nodal segment. However, MS medium supplemented with BAP (4.4 μM) in conjunction with NAA (0.53 μM) gave the best proliferation rate forming 19.8 ± 1.4 shoots after 60 days. This increase in shoot proliferation by addition of low concentration of auxin has earlier been reported in *D. strictus* (Ravikumar et al. 1998) and *B. nutans* (Negi and Saxena, 2011b).

5.1.5.1 Propagules

Propagule used for recurrent multiplication of shoots is a critical factor during *in vitro* studies. In the present study, shoot clumps having 3 shoots rather than single shoots were observed to be effective to achieve the increased rate of proliferation. However, use of more

number of shoots per clump (6, 9) reduced multiplication rate as well as length of shoots formed. In earlier studies also, shoot clumps rather than single shoots have been reported to be effective for multiplication of bamboo plants (Sood et al. 1992; Arya et al. 1999; Rathore et al. 2009 and Nadha et al. 2013). During micropropagation studies in *Dendrocalamus strictus* (Ravikumar et al. 1998) and in *Bambusa balcooa* (Negi and Saxena, 2011 a), the transfer of shoots in groups of 5-8 was recommended for sustained growth and multiplication. Agnihotri et al. (2009) reported 20-fold shoot multiplication rate with propagules of 3–5 shoots in *D. hamiltonii* while a cluster of 7-10 shoots was found optimum (supporting 5-6 fold multiplication rate) for large scale propagation of *D. asper* and *D. hamiltonii* (Singh et al. 2011, 2012a). Hence, shoot clusters yielded a consistent proliferation rate in maximum bamboo species.

5.1.5.2 Necrosis of Shoots

The problem of necrosis of shoots was of a common occurrence in all the three species under investigation that hindered *in vitro* growth leading to the death of bamboo regenerants. Medium turned brown due to accumulation of oxidized phenolic compounds released from multiple shoots after one month without sub-culture and resulted in necrosis of leaves and shoots. This necessitated the transfer of shoots to the fresh medium every 4-5 weeks. The necrosis of shoots have also been demonstrated by Nadgir et al. (1984); Paranjothy et al. (1990); Chaturvedi et al. (1993) and Huang et al. (2002) and many more. In *Oxyanthera abyssinica*, Diab and Mohamed, (2008) observed high necrosis of tissue at 7.0 and 4.0 mg/l of BA and NAA respectively and concluded that increased concentration of cytokinins and auxins in the media led to necrotic shoots. Hence, numerous factors lead to necrosis and so far not many reports are available to fully understand the cause of tissue browning.

5.1.5.3 Sucrose Concentration

In *D. membranaceus* and *B. bambos*, 2% sucrose was found to be most suitable carbon source forming 13.40 ± 1.5 and 21.70 ± 2.40 shoots respectively whereas in *B. balcooa*, 1% sucrose was found to be superior for better shoot proliferation with an average of 19.8 ± 1.4 shoots formed. In the present study, replacement of sugar with less expensive table sugar did not affect multiplication rate significantly, although it reduced cost of production considerably. Hence, table sugar was preferred over sucrose for carrying out micropropagation studies. Likewise, Singh et al. (2011, 2012a) reported that replacement of sucrose with less expensive table sugar had negligible effect on rate of shoot multiplication in *Dendrocalamus asper* and *D. hamiltonii*. Earlier also the addition of 2% sucrose has been advocated for best shoot proliferation in *Bambusa bambos* (Nadgir et al. 1984); *B. nutans* (Yasodha et al. 2008); *D.*

strictus (Shirgurkar et al. 1996 and Ravikumar et al. 1998); *D. hamiltonii* (Agnihotri et al. 2009) and *D. asper* (Nadha et al. 2013). In *B. balcooa*, medium incorporated with 3% sucrose resulted in albino shoots as it was found inhibitory for chlorophyll synthesis. However, the use of 3% sucrose has been recommended for shoot proliferation in numerous other bamboo species viz. *Dendrocalamus hamiltonii* (Sood et al. 1992); *D. giganteus* (Ramanayake and Yakandawala, 1997); *D. asper* (Arya et al. 1999); *Guadua angustifolia* (Jimenez et al. 2006); *Bambusa vulgaris* (Ndiaye et al. 2006); *B. glaucescens* (Shirin and Rana, 2007); *B. oldhamii* (Lin et al. 2007a) and *B. tulda* (Mishra et al. 2008) etc.

5.1.6 Rooting of Microshoots

For the development of any successful micropropagation protocol, root induction from excised shoots is a crucial factor. In bamboos, low rooting frequency has been regarded as the most serious bottleneck in developing a complete micropropagation protocol (Ali et al. 2009). Hence, understanding the hormone interactions in inducing rooting of microshoots in 'difficult to root' bamboo species holds importance. It was observed that rooting was more effectively induced when clusters comprising of 3-4 shoots rather than individual shoots were used as also reported by Sood et al. (1992); Bag et al. (2000); Agnihotri et al. (2009) and Nadha et al. (2013). Likewise, in *Bambusa bambos* (Arya and Sharma, 1998) and *Dendrocalamus hamiltonii* (Arya et al. 2012), single shoots failed to survive on rooting medium and thus, a propagules of minimum 3 shoots were required for effective rooting.

The role of auxins in root development is well established and concentrations and combinations of auxins required for inducing rooting vary with the species. Presently, in *D. membranaceus*, 5.37 μM of NAA when used in conjunction with 4.4 μM of BAP was found to be most effective for root induction with 65% rooting efficiency whereas Yasodha et al. (1997) achieved 73% rooting by using 0.5 mg/l IBA alone. In *D. brandisii*, Vongvijitra (1988) reported rooting in the presence of low concentration of BAP while carrying out micropropagation studies. BAP (0.5 mg/l) alongwith 0.2 mg/l Kn and 10% coconut milk has been employed for rooting in *Bambusa vulgaris* (Nadgir et al. 1984). In *B. ventricosa* (Huang and Huang, 1995); *Pseudoxytenanthera stocksii*, (Sanjaya et al. 2005) and *Arundinaria callosa* (Devi and Sharma, 2009), BAP (0.05 to 0.44 μM) alongwith auxins like IBA and NAA was used for best rooting percentage.

In our study on *Bambusa balcooa*, NAA (16.11 μM) when used alone was found to give best results for the root induction showing 76.6% rooting percentage. Our results are in agreement with the results of Sharma and Sarma (2011) who reported maximum number of roots with NAA (3.5 mg/l) alone in *B. balcooa*. On the other hand, Mudoj and Borathakur,

(2009) observed highest rooting in this species on MS medium supplemented with BAP and NAA while NAA (1-3 mg/l) alongwith IBA (1-5 mg/l) was employed for rooting by Islam and Rahman (2005). NAA has been consistently used for rooting in many other bamboo species like *Dendrocalamus strictus* (Mishra et al. 2001); *D. asper* (Arya et al. 2002) and *Bambusa oldhamii* (Lin, 2007 a) etc.

In the present investigation on *B. bambos*, rooting was difficult to achieve and has been a major problem to be worked out. Earlier, Nadgir et al. (1984) failed to achieve rooting in this particular species. Half strength MS medium fortified with 9.80 μM of IBA gave the best response for induction of roots with a root length of 7.04 ± 0.79 cm within 30 to 45 days in 60% of cultures followed by 5.37 μM of NAA depicting 48% rooting percentage. Further, addition of coumarin in the root inducing medium significantly improved the rooting response by 15 to 20 %. An initial pulse treatment of coumarin (9 mg/l) for 10 days and then shifting the cultures to coumarin free medium enhanced the rooting. Similarly, coumarin was used for enhanced rooting in *B. tulda* (Saxena, 1990); *Dendrocalamus longispathus* (Saxena and Bhojwani 1993) and *D. giganteus* (Ramanayake and Yakandawala, 1997). In *D. strictus*, Jimenez et al. (2006) reported root induction in 75% of cultures when shoots were given pulse treatment with coumarin (9 mg/l). Presently, continuous culture on medium with coumarin for relatively longer periods adversely affected growth and subsequent survival of shoots. IBA has also been used earlier in several bamboos for root induction as in *B. nutans* (Yasodha et al. 1997); *D. strictus* (Ravikumar et al. 1998 and Singh et al. 2001); *D. hamiltonii* (Agnihotri and Nandi, 2009) and *Melocanna baccifera* (Kant et al. 2009) etc.

In the present study, IBA and NAA proved to be effective for providing initial stimulus for *in vitro* rooting while advanced root growth was noticed only on transfer to auxin free medium. Therefore, the rooted microshoots were transferred to an auxin free medium for healthy growth of the roots as demonstrated earlier in *D. strictus* (Chaturvedi et al. 1993) and *D. hamiltonii* (Agnihotri et al. 2009) where low rooting efficiency occurred due to the inhibitory effects of auxins. A two step procedure was followed for rooting in *D. hamiltonii* (Arya et al. 2012; Bag et al. 2012 and Singh et al. 2012a) where a high rooting rate was reported when propagules were cultured on auxin supplemented medium for a week followed by their transfer to auxin free medium.

Shoots cultured on 1/2 MS medium gave better response among the three strengths tested (1/4, 1/2 and full) in *D. membranaceus* and *B. bambos*. This has earlier been reported in *D. membranaceus* (Yasodha et al. 1997); *D. strictus* (Reddy, 2006); and *D. asper* (Singh et

al. 2011 and Nadha et al. 2013). This has been attributed to reduction in total nitrogen required for rooting (Ajithkumar and Seenii, 1998).

In *Dendrocalamus strictus* and *D. hamiltonii*, incorporation of activated charcoal in the medium also induced rooting (Nadgir et al. 1984 and Sood et al. 2002 a). However, in the present study shoots turned necrotic and died on medium supplemented with charcoal. Attempts were made to induce rooting *ex vitro* but these efforts failed to achieve desirable results. Even pulse treatment of shoots with higher doses of auxins could not induce rooting *ex vitro*. In bamboos, differences in rooting performance may be attributed to their varied inherent genetic ability to root both *in vitro* and *ex vitro* conditions.

5.1.7 Acclimatization

The success of any micropropagation protocol depends upon the successful transfer and establishment of *in vitro* plants in the natural field conditions. Plants produced *in vitro* under controlled high humidity, diffused light and constant temperature need to be acclimatized before transferring them from *in vitro* to *ex vitro* conditions. It is therefore, necessary to transfer the plants to field through various hardening stages to increase the survival percentage. *In vitro* raised plants of *D. membranaceus*, *B. balcooa* and *B. bambos* were carefully rescued from the vessels and initially transferred to the plastic pots containing moist riverbed sand and covered with perforated plastic covers to maintain high internal humidity and kept in the growth room for a period of 14 days. Thereafter, they were transferred onto potting mixture containing sand: soil: farmyard manure (1:1:1) and shifted to green house to promote plant growth. In this period, the plants developed an efficient root system, built up new leaves and became photosynthetically active.

Type of potting mixture used during acclimatization is one of the important factors determining the survival percentage of the plants under *ex-vitro* conditions. Presently, plantlets of *B. balcooa* were successfully hardened off on potting mixture of soil: sand: FYM (1:1:1) where they showed the highest percentage survival (92.5%) as also advocated in *Bambusa glaucescens* (Shirin and Rana, 2007); *Arundinaria callosa* (Devi and Sharma, 2009); *Gigantochloa atroviolaceae* (Bisht et al. 2010); *Dendrocalamus hamiltonii* (Arya et al. 2012) and *Dendrocalamus asper* (Nadha et al. 2013). However in *B. balcooa*, Sharma and Sarma, (2011) recorded 100% survival rate when rooted plantlets were shifted to a mixture containing coco peat and vermicompost (3:1). In *B. bambos*, plantlets were hardened in soil: sand and FYM with more than 60% survivability whereas a mixture of soilrite: sand (2:1) was used by Arya and Sharma, (1998) for a period of 25 days and later plants were transferred to soil: organic manure (1:1) for better acclimatization. Compost was shown to be

the most suitable planting substrate for hardening which ensured high frequency survival (100%) of regenerated plants of *Guadua angustifolia* (Marulanda et al. 2002) whereas Jimenez et al. (2006) reported 85% hardening success using soil: sand and rice hulls. Using different potting mixtures like soil, sand, soilrite, perlite, vermiculite, compost or farmyard manure either alone or in various ratios, a survival rate of 70-95% have been reported in *D. strictus* (Nadgir et al. 1984); *D. asper* (Arya et al. 1999); *B. tulda* (Saxena, 1990); *B. arundinacea* (Arya and Sharma, 1998) and *B. nutans* (Negi and Saxena, 2011 b) .

In *B. balcooa*, only 30% of the rooted plants transferred directly to the potting mixture of sand: soil: manure (1:1:1) survived, while 90% survival was achieved when the plantlets were first shifted to sand covered with jars where high humidity around plants was maintained before transferring them to the potting mixture as also reported earlier by Saxena, (1990). In *B. arundinacea*; Arya and Sharma, (1998) regularly sprayed the plants with Hoagland solution after 1 week for healthy growth of plants as also done in the present study in all the three species. Devi and Sharma, (2009) sprayed half strength MS medium on *Arundinaria callosa* plants for better acclimatization. Season of field transfer has also been found to influence the survival rate and growth of plantlets and the months of July to August have depicted higher survival rate with sprouting of more new shoots than other months in *D. asper* (Singh et al. 2011). In the present study, it was observed that the best time for the transfer of hardened plants from green house to the field was the rainy season i.e. June end or July when adequate humidity prevail depicting 100% survival of transplanted plants.

5.1.8 Callus Induction and Proliferation

Induction of callus has been reported from different explants like inflorescences of *Bambusa oldhamii* (Yeh and Chang, 1986a); *B. beecheyana* (Yeh and Chang, 1986 b) and *B. edulis* (Lin et al. 2004 b); roots of *B. beecheyana* (Chang and Lan, 1995); leaves in *Phyllostachys viridis* (Rao et al.1990) and *B. multiplex* (Jullien and Van, 1994); anthers in *Sinocalamus latiflorus* (Tsay et al. 1990), and nodal segments in *Bambusa vulgaris* (Rout and Das, 1997; Mehta et al. 2010); *B. ventricosa* (Gielis, 1999); *B. nutans* (Mehta et al. 2010); *D. hamiltonii* (Godbole et al. 2002); *D. farinosus* (Hu et al. 2011) and internodal segments in *D. asper* (Shrotri et al. 2012). In the present study involving *D. membranaceus* and *B. bambos*, callusing could only be induced from internodal segments (0.5 to 1 cm) taken from *in vitro* raised shoots only. In *D. membranaceus*, callusing could also be initiated from seeds showing sustained growth. Callusing started at the cut ends of the explant after 15 days when incubated in the dark. Dark conditions were required for initiation of callus as also observed in *Dendrocalamus strictus* (Rout and Das, 1994). Incorporation of 2, 4-D (4.53 μ M) was

found to be essential for the induction of callus in *D. membranaceus* and *B. bambos*. It has been the most commonly used auxin for inducing nodular callus in bamboos (Mehta et al. 1982; Rao et al. 1985; Yeh and Chang, 1986a; Rout and Das, 1994; Godbole et al. 2002; Ogita, 2005; Arya et al. 2009; Mehta et al. 2010; Cheah and Chaille, 2011 and Bag et al. 2012). Many studies have confirmed that auxins like 2,4-D activate auxin response factors and auxin signal pathways and regulate genes related to growth and development (Tao et al. 2002 and Che et al. 2006) In present study, two types of calli were observed 1) nodular and compact 2) mucilaginous and transparent. Occurrence of such calli has also been reported earlier in *D. strictus* (Rao et al. 1985 and Rout and Das, 1994); *Sinocalamus latiflorus* (Yeh and Chang, 1987); *Otatea acuminata* (Wood et al. 1992); *D. longispathus* (Saxena and Bhojwani, 1993); *Bambusa beecheyana* (Chang and Lan, 1995) and *D. hamiltonii* (Godbole et al. 2002 and Arya et al. 2009) etc. Mucilagenous callus developed in the present study was not organogenic and consisted of fiber like cells as also observed in *B. vulgaris* (Saxena and Dhawan, 1999); *D. giganteus* (Ramanayake and Wanniarachchi, 2003) and *B. edulis* (Lin et al. 2004 a).

Strength of basic medium composition has been found to affect the callus growth in *D. membranaceus* and ½ strength MS medium favoured the callus proliferation. It has earlier been observed in *D. hamiltonii* (Godbole et al. 2002 and Bag et al. 2012) and *D. strictus* (Rout and Das, 1994) that ½ MS medium was better for the callus proliferation and organogenesis. It has also been indicated that sucrose concentration influences the overall growth of callus, browning and its proliferation (Woods et al. 1992). In *Dendrocalamus asper*, Arya et al. (2008a) found 2% sucrose to be best for callus growth while higher sucrose (6%) concentration retarded the callus growth and its multiplication. Similarly, in *B. edulis*, Lin et al. (2004 a) used 3-6% sucrose and found that 3% sucrose gave the best response for embryogenic callus proliferation. In contrast, Yeh and Chang, (1986 b) found 6% sucrose to be beneficial for callus proliferation in *B. beecheyana*. In *Dendrocalamus membranaceus*, best results regarding callus proliferation were obtained using 1% sucrose as also reported by Rao et al. (1985) in *D. strictus*. Myo-inositol has been included in the culture medium to stimulate the callus growth or induction of morphogenesis (Pollard et al. 1961). In the present study, it was found that 300 mg/l of inositol was the optimum concentration for best growth of seed callus. Likewise, the beneficial role of inositol for bamboo callus has earlier been demonstrated by Huang and Murashige, (1983).

5.1.9 Differentiation from Callus

It has been found that identity of tissues in the *in vitro* system is driven by ratio of auxins and cytokinins and affected by endogenous concentrations of these hormones within explant (Ali et al. 2009). Bamboos can regenerate via embryogenesis and organogenesis and the frequency of embryogenesis is lower than that of organogenesis (Zhang et al. 2010). Differentiation of callus can be improved by studying the events controlling transformation of nodular callus into shoot and root buds and finally converting them into plantlets (Singh and Seghal, 1999). Various combinations and permutations of auxins and cytokinins were tried for achieving regeneration from the callus in the present study.

In *D. membranaceus*, shoot bud differentiation was observed when callus was shifted to BAP (4.4 μM) and Kn (1.15 μM) supplemented medium in about 15% of cultures. These types of observations were also reported earlier in *D. strictus* (Mascarenhas et al. 1988) and *D. hamiltonii* (Zhang et al. 2010) etc. In *D. farinosus*, Hu et al. (2011) reported shoot regeneration from friable granular callus when transferred to MS medium supplemented with different concentrations of Kn (1.5 and 2.5 mg/l) alongwith IAA (0.5 mg/l).

In present study involving *Bambusa bambos*, shoot buds were formed on a medium supplemented with 2, 4-D (4.53 μM) and BAP (2.2 μM). In *Dendrocalamus hamiltonii*, Godbole et al. (2002) gradually decreased the concentration of 2, 4-D in the medium with corresponding increase in the concentration of BAP for increased regeneration from the callus. In *B. bambos*, lower concentration of 2, 4-D (2.26 μM) in conjunction with NAA (1.14 μM) resulted in the development of roots from callus lumps. In *D. giganteus*, Yasodha et al. (2010) reported rhizogenesis using 98.41 μM of IBA. There are only a few reports of somatic embryogenesis from seed callus in *Bambusa bambos* (Mehta et al. 1982 and Rao and Rao, 1988) while Vasana, (1985) used young leaves for achieving embryogenesis. In the present study, attempts to induce somatic embryogenesis remained unsuccessful. It is opined that the cells of bamboo are undoubtedly totipotent but some vital hormonal and/or nutritional factor or their combination for embryogenesis could not be discovered by us during the stipulated period of this project.

5.2 Seed Culture

After the pioneer report of Alexander and Rao, (1968) using seeds of *Dendrocalamus strictus* in establishing cultures, various workers have reported bamboo propagation through seeds/seedling explants (Nadgir et al. 1984; Vongvijitra, 1988; Saxena, 1990; Chaturvedi et al. 1993; Shirgurkar et al. 1996; Ravikumar et al. 1998; Arya et al. 1998, 1999; Singh et al. 2000; Bag et al. 2000 and Brar et al.2013). An *in vitro* propagation protocol using mature seeds of *D. membranaceus* and effects of different parameters on the germination of ageing

seeds was successfully carried out and is reported for the first time in this bamboo. The effects of sterilants, light conditions, exogenous applications of plant growth regulators and temperature in overcoming germination barriers in ageing seeds of this bamboo were studied. Control of contamination and establishment of aseptic cultures is a priority requirement because fungal and bacterial contamination is of recurrent occurrence in bamboos especially when seeds are used as explants as reported by Dekkers and Rao, (1989) and Shirgurkar et al. (1996). Fungal contamination was quite rampant in *D. membranaceus* seeds and seeds could be effectively sterilized using two different sterilants. Out of the various combinations of microbial disinfectants tried, treatment of seeds with 0.1% HgCl₂ and 15% sodium hypochlorite (NaOCl) for 10 minutes each gave the best results with 77.8 percent aseptic culture establishment. A combination of two sterilants at a moderate concentration for a long duration was effective rather than using high doses for a short time. This reveals that the endogenous contaminants affect the survival percentage of seedlings *in vitro* and prior selection and screening of initial planting material is imperative. Woods et al. (1992) reported a high level of contamination (30%) in *Otatea acuminata* when seeds were used as explants but contamination rates declined when embryos were excised from the seeds and cultured separately.

The role of PGRs in micropropagation of bamboos using seeds is well known (Saxena, 1990; Chambers et al. 1991; Maity and Ghosh, 1997 and Bag et al. 2000). PGR's in minute quantities are known to enhance the germination percentage and reduce the germination time (Vamil et al. 2010) is also evident in the present experiments involving the seeds of *Dendrocalamus membranaceus*. Vongvijitra, (1988) propagated the seeds of *D. membranaceus* and obtained the best results for multiple shoot proliferation using BAP at a concentration of 2×10^{-5} M. In *D. strictus*, Nadgir et al. (1984) multiplied the shoots derived from seedlings on MS medium supplemented with cytokinins. Presently, Kn alone has been found to be uncondusive and has minor regulatory role in seed germination and growth in *D. membranaceus*. The combination of two cytokinins (BAP and Kn) stimulated the growth of multiple shoots and allowed large number of plants to be produced from a limited supply of original plant material. Hence, in the present study, the active role of phytohormones in the regulation of seed germination was well established. Banik, (1987) also emphasized the importance of seed progenies as numerous plantlets could be produced through seeds under *in vitro* conditions for meeting the requirements for a variety of purposes. Hence, for species having available seeds, developing micropropagation protocols do holds importance. But many articles have highlighted the difficulty of obtaining the seeds because of long flowering

cycles in most bamboo species, poor seed set during sporadic flowering, short viability and consumption of their bulk amount by rodents as also observed under the present investigation. In the absence of seeds, at times it becomes difficult to raise plants through tissue culture even when the micropropagation protocols using seeds are available. On these accounts, propagation through seeds suffers badly.

Bamboo seeds germinate at higher percentage under shade than in direct sunlight. Thus bamboo seeds can be considered as negatively photoblastic (Banik, 1996). Light is an important variable and is not a requirement for initiating germination in some species including bamboos. Moreover, germination in this species is hypogeous as the kernel with the enclosed scutellum remains below the surface of the ground. Hence, dark conditions favoured germination initially. Many seeds have the minimum, the maximum, and the optimum temperatures at which they germinate. For example, tomato seeds have the minimum germination temperature of 50 °F and the maximum temperature of 95 °F, but an optimum germination temperature of about 80°F (<http://pubs.ext.vt.edu/426/426-001/426-001.html>). Regarding the role of temperature, the present results correspond with the above observation giving maximum germination at slightly higher temperatures. The optimum temperature after a lapse of 10 months or so was found to be 30°C in this species. Similarly, in *Arundinaria gigantea*, Baldwin et al. (2009) reported that the best germination of seeds occurred when a temperature regime of 35/25 °C or 30/20 °C was followed.

Ageing seeds are a difficult and complex system to be dealt with. In seeds of cereals, a certain degree of physiological dormancy (PD) generally exists (Savage and Leubner, 2006). Bamboo belonging to the same family does not exactly exhibit any dormancy but lose their viability very fast. Exogenous supply of GA₃ has been effective in overcoming dormancy to a certain degree in members of Poaceae family as presoaking seeds in a solution of GA₃ adds to the vigour of seeds. Our results showed that after a time lapse of 8-10 months, seeds did not respond to the treatment of phytohormones like BAP and Kn etc. The mean germination time (MGT) increased as older seeds contain a greater proportion of physiologically late germinating seeds and addition of GA₃ during culture initiation proved to be beneficial. Addition of GA₃ can increase the formation of rough endoplasmic reticulum and polyribosomes (Evins, 1971), besides its traditional role in increasing the embryo growth potential and endosperm weakening. In *Dendrocalamus strictus*, Maity and Ghosh, (1997) clearly established the potentiality of GA₃ in seed germination as well as clump formation from those of germinating seedlings. Biochemically, mobilization of food stored in the starchy endosperm is a post-germination event and gibberellins play an important role in the

production and secretion of hydrolytic enzymes from the aleurone layer (Taiz and Zeiger, 2003). Activity of the starch degradative enzyme β -amylase increases in the cotyledon and starch starts to decline as germination progresses (Dirk et al. 1999) and this also corresponded well with our results of starch test on germinating seeds.

Obtaining commercially useful seedlings from seeds is hindered by innumerable factors including uneven germination, low tolerance to desiccation, reduced seed longevity and hence viability. Ageing of seeds is progressive and leads to death of the seed and hence non viability (Schimdt, 2007). The present results using TTC showed approximately all seeds to be non viable after one year. Non viability of seeds has been tested earlier by TTC (Guzman et al. 2011) and is considered an important factor in seed trade, crop production and also in germplasm conservation and management. PGR treatments were found to be ineffective after certain time period which indicated the shelf life of seeds to be of 6-8 months and to the maximum of 1 year in the present study.

5.3 Evaluation of *In Vitro* plants and *Ex Vitro* Hardened Plants

5.3.1 Stomatal Studies

The study of physiological parameters provide valuable information regarding the capability of micropropagated plants for developing plantations. Tissue culture system is useful for the Evaluation of tolerance to environmental stresses as the stress conditions can easily be controlled *in vitro*. *Ex vitro* conditions were found to affect the stomatal density and stomatal pore length in all the three species under present investigation. In *Dendrocalamus membranaceus*, abaxial leaf surface of *in vitro* grown plant had a stomatal density of 25.33 ± 1.52 as compared to 29.33 ± 1.52 of *ex vitro* grown plant. Likewise, in *Bambusa balcooa* and *B. bambos*, *in vitro* grown leaves exhibited less number of stomata when compared to the leaves of hardened field established plants. Circular stomata changed shape to elliptical when facing water stress under *ex-vitro* conditions. Zaid and Hughes, (1995) reported that the stomatal frequency of *ex vitro* grown leaves of date palm was significantly higher than control plantlets growing in the *in vitro* environment. Similarly, Pospisilova et al. (1999) observed in *Nicotiana tabacum*, that the total number of stomata per leaf was more than double in *ex vitro* plants due to enormous leaf area growth after being transferred to *ex vitro* conditions.

5.3.2 Leaf Area Index

Leaf area at the beginning of the acclimatization phase was found to be an important characteristic for achieving a high leaf area at the end of acclimatization (Tadesse et al. 2001). In *Dendrocalamus membranaceus*, *ex vitro* hardened plants had LAI of 5.20 ± 0.65 as

compared to the *in vitro* grown plants showing a value of 1.35 ± 0.49 which correspond with the studies of Hirose et al. (1997) in *Ambrosia artemisiifolia* and *Abutilon theophrasti* where increased LAI of hardened plants was attributed to the increased CO₂ levels and rate of photosynthesis. Similarly, in *Bambusa balcooa* and *B. bambos*, a high leaf area index of 4.66 ± 0.81 and 4.78 ± 0.63 was exhibited in *ex vitro* plants when compared to that of *in vitro* grown plants. During the acclimatization phase, total leaf area increases gradually as it is not limited by the shortage of O₂ for respiration or CO₂ for assimilation of carbohydrates whereas under *in vitro* conditions the gas exchange in the sealed tubes/flasks and the inhibitory compounds accumulating in the tubes serve as the limiting factor for reduced growth (Cournac et al. 2002).

5.3.3 Relative Water Content (RWC)

The relative water content technique, formerly known as relative turgidity, was originally described by Weatherley, (1951) and has been widely accepted as a reproducible and meaningful index of plant water status. Dias et al. (2013) have demonstrated that immediately after *ex vitro* transfer of plants, the RWC decreased by 31% in *Ulmus minor*. Presently, the percentage of RWC was higher in the *in vitro* conditions owing to growth under controlled environmental conditions. In *D. membranaceus*, *B. balcooa* and *B. bambos*, *ex vitro* plants recorded a low RWC% of 56.6 ± 3.2 , 67.7 ± 3.3 , $50.5 \pm 3.0\%$ respectively as compared to the *in vitro* grown plants. RWC estimates in leaves is dependent upon changes in leaf variables such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration when plants face water stress under *ex vitro* conditions (Yamasaki and Dillenburg, 1999).

5.3.4 Electrolyte Leakage

Unstressed, undamaged plant cells maintain electrolytes within their membranes. The electrolyte efflux data supports that relative leakiness in acclimated plants is more as compared to the *in vitro* grown plants (Wilson and Jacob, 2003). Electrolyte leakage % of 76.0 ± 3.9 , 67.4 ± 2.4 and $70.15 \pm 2.5\%$ was recorded in *D. membranaceus*, *B. balcooa* and *B. bambos* respectively which had been found to be high as compared to the *in vitro* grown plants showing a value of 68.0 ± 2.9 , 57.7 ± 2.7 and $58.2 \pm 4.6\%$ respectively. This is due to high membrane permeability in outside environment. Whitlow et al. (1992) modified electrolyte leakage parameter as tissue ionic conductance which provided a more reliable measure of relative leakage and helped to know the membrane integrity of *Malus ioensis* and senescing leaves of *Quercus rubra*. Our studies showed positive influence of *in vitro*

conditions on relative water content and electrolyte leakage depicting the role of controlled conditions in maintaining the stability of biomembranes.

5.3.5 Chlorophyll Content

A comparative analysis revealed higher chlorophyll content in *ex vitro* hardened plants. In the present study, ultrasonics instead of conventional grinding was used for estimating the chlorophyll content as also supported by the results of Wu et al. (2002). The traditional method of chlorophyll extraction as used by Arnon, (1949) involves grinding the plant tissue in water miscible solvents, followed by centrifugation to remove solid material. These traditional procedures become noticeably time consuming especially when the number of samples is large. In the present study, efficiency of extraction was in the order acetone > DMSO > DMF due to differences in stability and viscosity of these solvents. Acetone exhibited the best extraction efficiency of chlorophyll in *ex vitro* plants of *B. bambos* showing value of 22.21 ± 2.1 mg/g fr.wt. followed by a values of 17.92 ± 1.33 mg/g fr.wt. and 17.25 ± 4.45 mg/g fr wt in *D. membranaceus* and *B. balcooa* respectively. Our report is in conformity with the work of Lin et al. (2013) who also demonstrated the higher efficiency of acetone for chlorophyll extraction in *Cinnamomum camphora*. In the present study, *ex vitro* transfer resulted in increased values of total chlorophyll content and carotenoids facilitating photosynthesis compared to *in vitro* grown culms.

5.3.6 Total Soluble Sugars (TSS)

Generally, plants accumulate some sort of organic and inorganic solutes in cytosol to raise osmotic pressure and thereby, maintain both turgor and driving gradient for water uptake. In *D. membranaceus*, *ex vitro* plants reported TSS value of 1.60 ± 0.32 mg/g fr.wt. as compared to 2.22 ± 0.13 mg/g fr.wt. in the *in vitro* plants. In *B. balcooa*, *ex vitro* plants recorded a value of 13.38 ± 2.03 whereas *in vitro* plants had a value of 16.50 ± 0.78 mg/g fr.wt. and in *B. bambos*, sugar content was found to be 12.00 ± 2.2 mg/g fr.wt. in *ex vitro* grown plants as compared to 16.1 ± 1.0 mg/g fr.wt. values in the *in vitro* grown plants. Similar findings have been reported by Saini et al. (2012) in *Camellia* spp. The reason for the lower values of sugars in *ex vitro* hardened plants is attributed to the higher utilization of sugars for growth and metabolism growing in the autotrophic conditions as compared to the artificial conditions of the culture room. Using $^{14}\text{CO}_2$, Hansen, (1971) estimated that until the development of the first five to six leaves for extension shoots, about one-half to two-third of the building material comes from reserve.

5.3.7 Starch Content

Generally, starch serves as the dominant storage carbohydrate in higher plants. Aracama et al. (2006) demonstrated that starch utilization correlated with the development of leaves and expanded leaf blades during *ex vitro* hardening which has also been observed in the present study for all the three species. In *D. membranaceus*, *B. balcooa* and *B. bambos*, *ex vitro* hardened plants depicted a low values of starch i.e. 3.65 ± 0.15 , 4.76 ± 0.51 and 2.02 ± 0.1 respectively whereas *in vitro* plants have shown comparatively higher values of 3.90 ± 0.14 , 6.32 ± 0.55 and 2.33 ± 0.1 mg/g fr.wt. respectively. A common strategy of plants during *ex vitro* acclimatization is to use the carbohydrate storage of *in vitro*-produced leaves to cover metabolic demands of growing tissues (Fuentes et al. 2005). The overall low levels of starch under *ex vitro* conditions may be due to a general increase in total metabolic activity caused by autotrophic conditions. It is also possible that more rapid hexose metabolism is involved (Prado et al. 2000). This assertion was further supported by studies with a variety of plants showing a salt or drought-induced conversion of hexoses and other carbohydrates such as sucrose and starch into sugar alcohols (polyols) and proline (Perez-Alfocea and Larher, 1995 and Wang et al. 1998).

5.3.8 Proline Content

Plants adapt to stresses by different mechanisms, including changes in morphological and developmental patterns as well as physiological and biochemical processes (Bohnert et al. 1995). It has been widely reported that plant cells achieve their osmotic adjustment by the accumulation of some kind of compatible solutes (osmoprotectants) such as proline, betaine and polyols to protect membranes and proteins (Gorham et al. 1981 and Ashraf and Foolad, 2007). In the present study involving *D. membranaceus*, *B. balcooa* and *B. bambos*, *ex vitro* plants had a proline content of 2.68 ± 0.36 , 2.47 ± 0.28 and 2.94 ± 0.2 $\mu\text{M/g}$ respectively which has been found to be higher as compared to the proline content of *in vitro* grown plants. Plants resort to many adaptive strategies due to their sedentary mode of life, and proline accumulation being one of them, in response to different abiotic stresses such as high salt, dehydration, heat and excessive osmotic pressure, which ultimately affect plant growth and productivity (Yancey et al. 1982).

5.3.9 Lignin Content

According to Lin et al. (2002), lignification increases with ageing but in contrast Itoh, (1990) stated that lignification is completed within one growing season. In the present investigation, in *D. membranaceus*, *ex vitro* hardened plants showed a lignification value of 29.50 ± 1.50 mg/l as compared to *in vitro* grown plants showing a value of 25.02 ± 0.65 mg/l; in *Bambusa*

balcooa, *ex vitro* plants showed a value of 28.74 ± 1.59 mg/l against *in vitro* values of 17.90 ± 2.16 mg/l and in *B. bambos*, *ex vitro* plants had lignification of 41.2 ± 3.1 mg/l as compared to *in vitro* grown plants of 33.13 ± 4.1 mg/l etc. According to the earlier work of Mckendry (2002), the *ex vitro* hardened plants had more pronounced growth due to photoautotrophic mode of photosynthesis and hence, more biomass in term of lignin content as is well supported by our studies as well.

5.3.10 Anatomical Studies

Before exploiting any plant for industrial application, it is imperative to have complete information about its biology, chemistry, anatomy and all other applications so that the potential of plant could be utilized maximally (Garg et al. 2011). Hence, study of internal anatomy was carried out in the present thesis. Differences in the anatomy and microstructures in the present bamboo species especially in epidermal layers of culm and root, collateral vascular bundles, vasculature around midrib in leaf, size and shape of fusoid cells and bulliform cells were observed as has also been reported by Mustafa et al. (2011) in several species of *Gigantochloa*. The anatomy and the physical properties of bamboo culms are known to have significant effects on their durability and strength and will be helpful in determining their possible proper utilization (Razak et al. 2011). Poaceae leaf epidermis has intercostal and costal zones as also observed by Metcalfe, (1956) which further consists of long and short cells. Our investigations corresponded well with studies of Vieira et al. (2002) that bamboo midrib consists of complex vascular system, mesophyll consisting of tabular lobed chlorophyllous elements and fusoid cells, vascular bundles with double sheath, epidermis made up of long cells and short cells, micro hairs, prickles and silica bodies corresponding to the 'bambusoid type' of leaf anatomy. The presence of epidermal cells having silica bodies is an important character in Poaceae family (Metcalfe, 1963). Prickles have already been mentioned in Bambusoideae species by Soderstrom and Ellis, (1988). There is, however, no comprehensive publication available on the anatomy of bamboo culm, with the available literature being fragmented, scattered and inadequate. Guerrerio et al. (2013) worked on culm anatomy of Andean woody bamboos and some features like epidermis, hypodermis, sclerenchyma and position of phloem in vascular bundles was revealed. Bambusoid root anatomy has received scant attention to date. Raechal and Curtis, (1990) also studied root anatomy in 15 species in 6 of the 11 tribes of Bambusoideae. They reported that roots of species show some variations among each other but they mostly resemble panicoid type. These variations could play role in characterization of taxa within tribes.

5.4 Clonal Fidelity

The selection of elite genotypes is at the basis of clonal forestry and occurrence of somaclonal variations is a potential drawback when propagation of an elite plant species is intended. It is presumed, in general, that the *in vitro* derived plants are exactly similar to the mother plant. However, when plant tissue is passaged through *in vitro* culture, many of the regenerated plantlets appear to be no longer clonal copies of their donor genotype, due to genetic changes (Larkin and Scowcroft, 1981). The broader utility of any micropropagation system may be hindered due to the occurrence of cryptic genetic changes and development of somaclonal variants (Rani and Raina, 2000 and Kaeppler et al. 2000). Many factors are responsible for inducing variability during tissue culture such as explants source, time of culture, number of subcultures, phytohormone, genotype, media composition, the level of ploidy and genetic mosaicism (Silvarolla, 1992). The activation of transposable elements (Hirochika et al. 1996), DNA hypomethylation (Keyte et al. 2006), genome adaptation to different regulatory microelements (Bogani et al. 1996) and the presence of hot spots (Linacero et al. 2000) are major mechanisms expected to induce above variations.

Although several reports are available on the micropropagation of bamboos but there are only a few reports available ascertaining the genetic fidelity of bamboo plantlets derived from axillary bud proliferation. The scarcity of reports on confirming the clonal fidelity of tissue culture raised plantlets can jeopardise the quality of micropropogated plants, especially in perennials like bamboos where any undesirable variant would last for several years (Negi and Saxena, 2010). Therefore, it is pertinent to screen the regenerants at regular intervals for the occurrence of any somaclonal variation (Chandrika et al. 2008). Use of molecular markers for precise identification, to assess natural variability and for early assessment has been highlighted by Gielis et al. (1997). RAPD and ISSR dominant markers were selected in the present study due to their technical simplicity, high speed, low cost and user friendliness. RAPD markers are quick and have a relatively economical approach to detect even small genetic differences as has also been observed by Friesen and Klaas, (1998) and Bhagat et al. (2012). ISSR markers as described by Zietkiewicz et al. (1994) use microsatellite oligonucleotides as primers that amplify genomic segments different from repeat region itself (Negi and Saxena, 2010). Some reports have suggested the use of more than one DNA amplification technique as being advantageous for evaluating genetic stability of micropropagated plantlets in several crops like almond (Martins et al. 2004), banana (Ray et al. 2006), *Dendrocalamus hamiltonii* (Agnihotri et al. 2009); *D. asper* (Nadha et al. 2013)

and *Guadua angustifolia* (Nadha et al. 2011) which is also in consonance with our results where both RAPD and ISSR assays have been used to assess the genetic fidelity.

Till date, there is no report available on the comparative genetic stability of regenerants and mother plant of *D. membranaceus* and *B. bambos* by using RAPD and ISSR markers. Presently, in *D. membranaceus*, during RAPD analysis, out of 35 primers screened, 30 primers produced clear and reproducible bands. These 30 RAPD primers produced 73 distinct and scorable bands in the size range of 200 bp to 1500 bp. *In vitro* raised plants of *Bambusa balcooa* through the axillary bud proliferation were scanned with 21 RAPD primers and produced 61 distinct and scorable bands in the size range of 100 bp to 1500 bp. In *B. bambos*, out of 15 RAPD markers scanned, only 10 primers produced amplicons. In case of ISSR analyses, using 10 markers, only 5 markers gave scorable bands with no variations reported in the progeny. Earlier, Das and Pal, (2005b) established the genetic uniformity among the regenerants of *Bambusa tulda* and *B. balcooa* using only four RAPD markers. Later, Negi and Saxena, (2010) employed 15 ISSR markers to validate the clonal fidelity of *Bambusa balcooa* plants multiplied by enhanced axillary proliferation up to 33 passages. Godbole, (2003) assessed the clonal fidelity of *in vitro* raised plants of *D. hamiltonii* using 18 RAPD markers. In *B. pallida*, a total of 25 RAPD primers were scanned depicting no variation in the micropropagated plants (Beena and Rathore, 2012). In *B. nutans*, Mehta et al. (2010) used AFLP markers and obtained four hundred and seven scorable fragments, of which 402 (98.8%) have recorded conservation in regenerated plantlets, therefore, revealing a high level of genetic stability.

5.5 Field Performance of *In Vitro* Raised Plants

The benefit of any micropropagated system can be fully realized only by the successful establishment of the plants to *ex vitro* conditions. After an *ex vitro* transfer, the plants get easily impaired by sudden changes in environmental conditions and need a certain period for adaptation to increased irradiance, low relative humidity (RH) and changes in the water potential (Hazarika, 2003). Only a few reports are available regarding successful field transfer of micropropagated plants in bamboos and still fewer regarding their field performance studies. Tissue culture raised plants of all the three species were first kept in high humid conditions of the green house for 5-6 months for initial acclimatization. The best time for their transfer to the fields was the beginning of rainy season i.e. June end to July where they exhibited 100% survival. The small plants would not survive if transplanted directly during adverse conditions of winter in the natural open environment. The most probable reason for large mortalities in tissue culture raised plants transplanted during winters

could be due to the fact that all these three species under investigation are tropical bamboos, inhabitants of warm areas and therefore more susceptible to low temperatures as also shown earlier in *Dendrocalamus strictus* (Saxena and Dhawan, 1999).

Field performance of *in vitro* raised plants under field conditions was recorded by studying different plant parameters like height, number of shoots, number of leaves, number of axillary shoots per clump and number of internodes per shoot and the data was recorded for the six months under field conditions. *D. membranaceus* registered a maximum growth in terms of height achieved i.e. 101.66 cm, 294 leaves formed and on an average 24.6 new shoots emerged during this period. *Bambusa balcooa* showed maximum height of 95.5 cm, 255 leaves and 20 new shoots formed while in *B. bambos*, height of 50 cm was achieved with 94 leaves and 12 new shoots formed. In *Dendrocalamus hamiltonii*, Agnihotri et al. (2009) reported 70% survival in the field and observed six fold increments in plant height in 18 months. In *Bambusa nutans*, Negi and Saxena, 2011 b achieved 100% survival and the culms attained the height of 2.02m within 9 months of transplantation to the field. Devi and Sharma, (2009) established the plants of *Arundinaria callosa* with 60-70% efficiency, whereas in *Dendrocalamus asper*, 95% survivability was reported in the fields (Arya et al. 1999). Rathore et al. (2009) have demonstrated that out of *Bambusa bambos*, *B. balcooa*, *Dendrocalamus stocksii* and *D. asper*, the field performance of *B. balcooa* proved to be the lowest in various field trials in Bangalore. Islam and Rahman, (2005) recorded the field performance of six bamboo species depicting 100% survival and good growth vigour after a period of nine months.

Conclusion

1. The development and standardization of an efficient and reproducible protocol for large scale propagation through forced axillary branching has been successfully achieved in three important edible bamboo species namely *Dendrocalamus membranaceus*, *Bambusa balcooa* and *B. bambos*.
2. The results revealed the significance of nodal explants in maintaining the prolonged *in vitro* cultures with true to type progenies. The effectiveness of the micropropagation protocol was dependent upon the season, size of explant, plant growth regulators and on successful acclimatization of the plantlets.
3. Sucrose was found to significantly alter the efficiency of *in vitro* propagation depending upon the type of species. In *B. balcooa*, best shoot proliferation occurred on 1% sucrose, whereas 2% sucrose was mandatory for highest shoot proliferation in *D. membranaceus* and *B. bambos*.
4. The use of auxin (NAA) alongwith cytokinin (BAP) in shoot multiplication is also a new finding in *Bambusa balcooa*.
5. *In vitro* propagation protocol using mature seeds of *D. membranaceus* is established and reported for the first time where GA₃ was found to be effective in increasing germination percentage at higher temperature in ageing seeds.
6. Physiological studies conducted in the course of this study i.e. relative water content (RWC), electrolyte leakage, stomatal density and leaf area index (LAI) helped understand the transition of plantlets from the *in vitro* conditions to the soil. RWC was found to be more in the *in vitro* raised plants whereas electrolyte leakage, stomatal density and LAI were found to be more in the hardened plants in all the species under study.
7. Biochemical parameters like lignin was found to be higher in acclimatized plants due to more pronounced growth and metabolism whereas total soluble sugars (TSS) and starch contents were lower in all the three species. Proline content was reported to be higher in *ex vitro* plants as it helped in maintaining cellular homeostasis by acting as a compatible osmolyte to repair stress induced damage.

8. The extraction of chlorophyll from the epidermis of culms using acetone, dimethylformamide and dimethylsulfoxide as solvents is reported for the first time in *D. membranaceus* and *B. balcooa* where increased level of CO₂ in green house promoted the synthesis of chlorophyll.
9. Establishment of clonal fidelity of the *in vitro* raised plants and the mother plant using RAPD and ISSR markers to ensure homogenous progenies in all the three species was achieved. All the bands scored were monomorphic across the mother plant and its tissue culture raised progenies, thereby, indicating genomic conservation during *in vitro* multiplication. This is the first report on the comparative genetic stability of regenerants and mother plant of *D. membranaceus* and *B. bambos* by molecular markers.
10. The field performance in terms of height of plant, number of shoots, number of leaves, number of axillary shoots per clump and number of internodes per shoot for all the three species was successfully recorded indicating an increase in overall growth parameters of plants in open field conditions.
11. Micropropagation protocol developed will ensure regeneration of large number of plants in a relatively short time and ensures conservation of these economically important edible bamboo species. Hence, with the ever increasing global inclination towards the alternate sources of raw material for pulp and sustainable development of food, the present study fills gaps in knowledge base of bamboos and offers a wide scope for mass cloning of these multipurpose plants.

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Research Publications

1. **Jasmine Brar**, Amrina Shafi, Priyanka Sood, Manju Anand and Anil Sood (2014) *In vitro* propagation, biochemical studies and assessment of clonal fidelity through molecular markers in *Bambusa balcooa*: an important edible bamboo. *Journal of Tropical Forest Science*, 26(1), 115-124.
2. **Jasmine Brar**, Manju Anand and Anil Sood (2013) *In vitro* propagation of economically important edible bamboo *Dendrocalamus membranaceus* Munro. *Indian Journal of Experimental Biology*, 51(1), 88-96.
3. Manju Anand, **Jasmine Brar** and Anil Sood (2013) *In vitro* propagation of an edible bamboo *Bambusa bambos* and assessment of clonal fidelity through molecular markers. *Journal of Medical and Bioengineering*, 2(4), 257-261.
4. **Jasmine Brar**, Amrina Shafi, Priyanka Sood, Anil Sood and Manju Anand (2012) Micropropagation of *Dendrocalamus membranaceus* Munro. through axillary shoot proliferation and ascertaining clonal fidelity of *in vitro* raised plants. *Journal of Bamboo and Rattan* (Accepted).

National / International Conferences

1. **Jasmine Brar**, Manju Anand and Anil Sood (2014) Comparison of biochemical parameters in the *in vitro* and *ex vitro* plants of *Dendrocalamus membranaceus*. 17th Punjab Science Congress, Punjab Technical University, Jalandhar.
2. Manju Anand, **Jasmine Brar** and Anil Sood (2013) *In vitro* propagation of an edible bamboo *Bambusa bambos* and assessment of clonal fidelity through molecular markers. International Conference on Biotechnology and Food Engineering (ICBFE-2013), Singapore.
3. Manju Anand, **Jasmine Brar** and Anil Sood (2013) Comparison of chlorophyll content in *in vitro* and *ex vitro* explants of *Dendrocalamus membranaceus* using different solvents by ultrasonics. 5th National Conference on Recent Advances in Chemical and Environmental Sciences (RACES – 2013), Multani Mal Modi College, Patiala, 31st January, Pp. 59.
4. **Jasmine Brar**, Manju Anand and Anil Sood (2013) Micropropagation of edible bamboo (*Bambusa bambos*) and assessment of clonal fidelity through molecular markers. 16th Punjab Science Congress, Baba Farid University of Health Sciences, Faridkot, February 7-9, Pp. 169.
5. Manju Anand, **Jasmine Brar** and Anil Sood (2012) Micropropagation of an important bamboo *Dendrocalamus membranaceus* and evaluation of clonal fidelity of micropropagated plants using RAPD and ISSR markers. National Seminar on Plant Cell Tissue and Organ Culture- Emerging Trends, AMU, Aligarh, Pp.16.