# *In vitro* clonal propagation of some important woody bamboos and ascertaining their clonal fidelity

***A thesis submitted in fulfilment***

***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 "*In vitro* clonal propagation of some important woody bamboos and ascertaining their clonal fidelity" in fulfilment of the requirement for the award of the degree of Doctor of Philosophy at the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period from July 2008 to February 2012, under the supervision of Dr. Manju Anand, Assistant Professor, Department of Biotechnology and Environmental Sciences, 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.

Harleen Kaur

Harleen Kaur Nadha

Place:Patiala

Date: 03.3.2013

# Certificate

Certified that the thesis entitled "***In vitro* clonal propagation of some important woody bamboos and ascertaining their clonal fidelity"**, submitted by Ms. Harleen Kaur Nadha, in fulfilment of the requirement for the award of **Degree of Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, 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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Harleen Kaur

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# Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxy acetic acid |
| ABA | Abscissic acid |
| BAP | Benzylaminopurine |
| bp | basepair |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| °C | Degree celsius |
| CM | Coconut milk |
| cm | Centimeter |
| conc. | Concentration |
| D.P.X | Distyrene plasticizer xylene |
| FAA | Formaldehyde: Acetic Acid: Alcohol |
| ft | Feet |
| g | Gram(s) |
| g/l | Gram(s) per litre |
| GA3 | Gibberellic acid |
| h | Hour(s) |
| ha | Hectare(s) |
| HCl | Hydrochloric Acid |
| HgCl2 | Mercuric Chloride |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| Kn | Kinetin |
| lb/ $in^2$ | Pound per square inch |
| KPa | Kilo pascal |
| kb | Kilo base pair |
| m | Meter |
| M | Molar |
| mg | Milli gram |
| min | Minute(s) |
| ml | Millilitre(s) |
| MS | Murashige and Skoog's (1962) medium |
| msl | mean sea level |
| µM | Micromole |
| µl | Microlitre |
| NAA | Napthalene acetic acid |
| NaCl | Sodium Chloride |
| O.D. | Optical Density |
| PEG | Polyethylene Glycol |
| PCR | Polymerase chain reaction |
| PGR | Plant Growth Regulator |
| PVP | Polyvinyl pyrrolidone |

| | |
|---|---|
| % | Percent |
| rpm | Revolution per minute |
| PVP | polyvinyl pyrrolidone |
| RAPD | Random Amplified Polymorphic DNA |
| Taq Pol. | Taq DNA polymerase |
| TBA | Tertiary butyl alcohol |
| TBE | Tris borate EDTA |
| TDZ | Thidiazuron |
| TE | Tris-EDTA |
| UV | Ultra-violet |
| v/v | Volume by volume |
| WPM | Woody Plant Medium of Lloyd and McCown (1982) |
| w/v | Weight by volume |

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# 4 Results

## Objective 1

To develop and standardize mass propagation of *Bambusa tulda*, *Dendrocalamus asper, Guadua angustifolia*, *Phyllostachys pubescens* under *in vitro* conditions.

**Objective 2**

To test the clonal fidelity of tissue culture raised plants by RAPD and to study their field performance.

## 5 Discussion

# Chapter 1
# Introduction

# 1 Introduction

## 1.1 Bamboos: An overview

Bamboos are the most important forest species of Southeast Asian countries where they form the backbone of rural economy. They are rightly called the “Green Gold of Forests” on account of their fast growth, short rotation cycles, multiple uses, little or no after plantation care and multiple industrial uses, thereby, providing employment to millions of people. Bamboos have more than 1500 recorded uses. According to an estimate, 2-5 billion people depend on or use bamboo material to a value of 7 billion US $ per annum (Liese, 1991). Over 70 genera of bamboos with over 1200 species occur in natural forests, semi exploited strands and in intensive plantation covering an area of more than 14 million hectare worldwide (Dransfield and Widjaja, 1995). China with the largest bamboo industry worldwide has a total of about 7 million hectare of bamboo forests (Perez et al, 1999). The area under bamboos in India has been estimated between 3 and 10 million hectare (Biswas, 1998). China ranks first with 26 genera and 300 species while India having 23 genera and 125 species occupies second place and produces 4-6 million tonnes of bamboos annually, out of which 1.9 million tonnes are used in the paper industry. Besides their application in paper industry, they are extensively used for house building, furniture making and floor tiles. Bamboo shoots are one of the most widely consumed delicacies in Asia (Tripathi, 1998). Shoots can be consumed fresh, cooked, pickled, fermented and canned (Nirmala et al. 2008). The leaves of bamboos have been used in Asian countries as a food wrapping material to prevent food deterioration since ancient times. Leaves also serve as an excellent cattle fodder especially during winter months when there is a shortage of green fodder. The leaves have also been utilized clinically in the treatment of hypertension, arteriosclerosis, cardiovascular diseases

and certain forms of cancer. In the tropics, bamboo is a very important plant, providing livelihood for over 500 million people and providing housing and shelter for over 1 billion people. Besides supplying products of immediate use to humans, bamboos also render multiple ecological services such as soil and water conservation, erosion control and act as a significant sink for global carbon dioxide. Due to their over utilization and lack of commercial cultivation, there is a rising concern about acute scarcity of bamboo products in the near future (Hsiung, 1988).

**1.2 Growth and development of bamboos**

Bamboos comprise a subfamily Bambusoideae of the grasses (Poaceae). They are evergreen, monocotyledonous plants which produce primary shoots without any secondary growth. Each 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. Young culms with compressed internodes and including a part of the culm neck are harvested for edible shoots, weighing from 0.25 to 500 kg each depending on the species. Mature culms provide timber that is put to multiple uses. Rhizome consists of nodes and internodes.

According to their morphology, bamboos are broadly divided into monopodial (or running) bamboos with “leptomorph” rhizome system and sympodial (or clumping) bamboos with “pachymorph” rhizome system. The internodes of pachymorph rhizome are broader than long and lateral buds on nodes produce only rhizomes (Valade and Dahlon, 1991). In sympodial bamboos, new culms develop from buds on elongated culm necks (pseudorhizomes) rather than from buds on rhizomes. 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. The tight-clumping habit of tropical species supposedly evolved from the leptomorph form of rhizomes which provides less rhizome surface to dehydrate during extended dry seasons (Farrelly, 1984).

The phrase that bamboo is “one of the fastest growing plant” is attributed to the speed of culm growth. This fast growth phase results from expansion of individual internodes and depending on species, culms can grow up to 3-30m height within 3-4 months (Liese and Weiner, 1995). It is generally agreed that up to a certain age, height and diameter of the annual flush of the culm increases. Particular environmental conditions such as higher temperature, greater water availability and higher air humidity promote culm growth. Culms which emerge early in the season can fully develop during the warm summer in the temperate climate and during the wet season in tropical climate, where as “late” culms rarely survive in the second growing season due to cool-temperature conditions in temperate climate or to dry soil conditions in tropical climate (Pearson et al. 1994).

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. Heterophylls are intermediates between culm sheath and foliar leaves. The number of heterophylls is characteristic of the species. Bamboo flowering is an enigma even today. The flowering is erratic and occurs at relatively long intervals, but at the same time sporadic flowering in some culms is not uncommon. Otherwise, the flowering periods for each species are generally fixed. The culms / clumps generally dry after flowering but there are instances when the clumps are found to have rejuvenated at a later

stage. Bamboos, however being cross pollinated, their seed progenies show greater heterozygosity and may not be suitable for large scale plantation.

### 1.3 Bamboos and Plant Tissue Culture

In view of the growing world population, increasing anthropogenic activities, rapidly eroding natural ecosystems, the natural habitats for a great number of bamboo species are dwindling. Other factors responsible for the depletion of bamboo resources are : i) lack of scientific management of existing strands ii) inadequate efforts to replenish natural bamboo forests and raise new plantations iii) as compared to other forest species, bamboos have rather received scant attention. In India, the states of Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, Tripura and West Bengal are rich in bamboo diversity but over-exploitation in the past has led to the scarcity of raw material even in these states.

Most of the bamboo species do not produce seeds. Moreover, propagation through seeds is unreliable because of long flowering cycles (generally 30 to 60 years), short seed viability and extremely poor seed-set during sporadic flowering and consumption of good amount of seeds by wild animals, especially rodents. The traditional propagation method by ‘offsets’ limits the number of propagules and again it is a slow, cumbersome and highly labour intensive procedure for large scale establishment of bamboo plantations. 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. In this regard, micropropagation has emerged as a promising technique for rapid and mass production of genetically pure elites rather than having indifferent populations.

**1.4 Advantages of Micropropagation**:

1. Micropropagation can be used as an alternative to conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication.
2. Through *in vitro* clonal propagation, a large number of plants can be raised from even small sized explant within a short span of time.
3. Micropropagation provides reliable and economical method of maintaining pathogen free plants in a state that can allow rapid multiplication and also facilitates exchange of germplasm and transportation.
4. Plant multiplication can continue throughout the year irrespective of season.
5. Stocks of germplasm can be maintained for many years.

## 1.5 CLASSIFICATION OF BAMBOOS

| | | |
|---|---|---|
| KINGDOM | - | Plantae |
| RACE/CLASS | - | Monocotyledons |
| ORDER | - | Poales |
| FAMILY | - | Poaceae |
| SUB-FAMILY | - | Bambusoideae |
| SUPERTRIBE | - | Bambusodae |
| TRIBES | - | Bambuseae |

SUBTRIBES

| Racemobambodinae | Shibataeinae | Arthrostylidiinae | Arundinariinae | Bambusinae | Melocanninae | Nastinae | Guaduinae | Chusqueinae |
|---|---|---|---|---|---|---|---|---|
| Racembobambos | Brachystachyum | Actinocladum | Acidosasa | Bambusa | Cephalastachyum | Decaryochloa | Apoclada | Chusquea |
| | Chimonobambusa | Alvimia | Ampclocalamus | Bonia | Davidsea | Greslania | Eremocaulon | Neurolepis |
| | Indosasa | Arthrostylidium | Arundinaria | Dendrocalamus | Leptocanna | Hickelia | Guadua | |
| | Phyllostachys | Athroostachys | Borinda | Gigantochloa | Melocanna | Hitchcockella | Olmeca | |
| | Qiongzhuea | Atractantha | Chimonocalamus | Dinochloa | Neohouzeaua | Nastus | Otatea | |
| | Semiarundinaria | Aulonemia | Drepanostachyum | Holltuncochloa | Ochlandra | Perrierbambus | | |
| | Shibataea | Calanthelia | Fargesia | Kinabaluchloa | Pseudostachyum | | | |
| | Sinobambusa | Elytrostachys | Ferrocalamus | Melocalamus | Schizostachyum | | | |
| | Temburongia | Glaziophyton | Gaoligongshania | Sphaerobambos | Teinostachyum | | | |
| | | Merostachys | Gelidocalamus | Thyrsostachys | | | | |
| | | Myriocladus | Himalayacalamus | | | | | |
| | | Rhipidocladum | Indocalamus | | | | | |
| | | | Olgostachyum | | | | | |
| | | | Pseudosasa | | | | | |
| | | | Sasa | | | | | |
| | | | Phamnocalamus | | | | | |
| | | | Yushania | | | | | |

GENUS

### 1.6 Plant profile

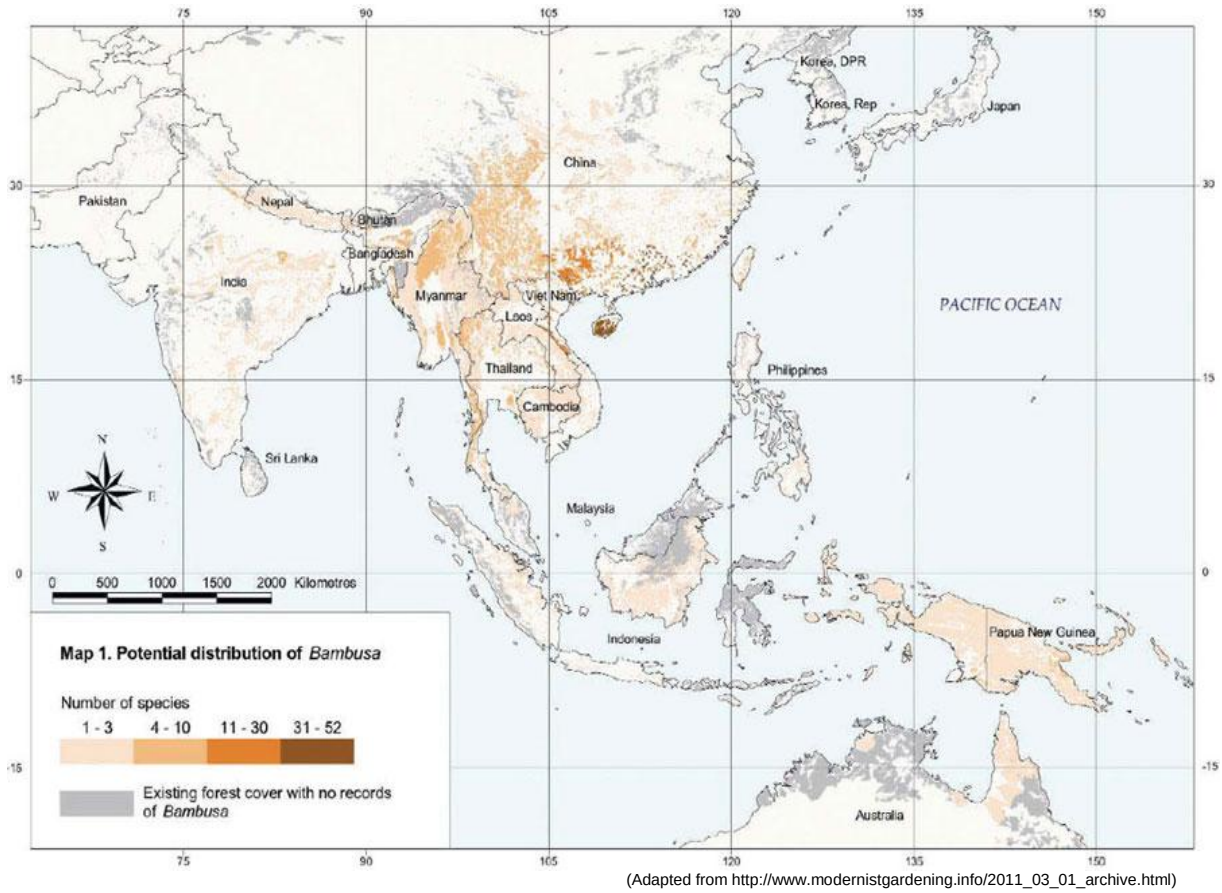
It was proposed to carry out micropropagation studies on four bamboo species namely *Bambusa tulda*, *Dendrocalamus asper*, *Guadua angustifolia* and *Phyllostachys pubescens*.

#### 1.6.1 *Bambusa tulda* Roxburgh

It is commonly known as Bengal bamboo, Spineless Indian bamboo or Calcutta bamboo.

**Distribution-** It is found in biome of Southeast Asian rainforests. It often grows as undergrowth, scattered or in patches in the forests. In India**,** it is found extensively in North-East and West Bengal and is cultivated in Arunachal Pradesh up to an altitude of 1000m and in the Brahmaputra valley. It is also cultivated in Uttar Pradesh, Uttrakhand, Karnataka and West Bengal (Figure 1.1). **Habitat-** It occurs at an altitude up to 1500m and thrives along water courses. It prefers good rainfall areas with moist alluvial soil that is rich in organic matter, nitrogen, calcium, potassium and phosphorous. The pH preference is 4.5 to 6.5. It also grows in fine-textured soils in semi-evergreen forests in relatively low rainfall areas. **Culm**- The culm is up to 25 m tall, thick-walled, strong, upright and smooth (Figure 1.2). The young culm is dark green in colour with a slight whitish bloom on the internodes that comes off easily. It is greyish-green when old and sometimes the 2-4 basal nodes have whitish yellow stripes. Internodes are 40 to 70 cm long, 5 to 10 cm in diameter and have wall thickness of 0.8 to 1.5 cm. The lower nodes have fibrous roots. Branches emerge from the lower part and are closely packed. At each culm node, there are usually three large branches and many small branches (Figure 1.3). **Leaves-** The leaves are long and narrow and green in colour. They grow alternatively on opposite sides of the stem, in two rows (Figure 1.4). **Culm sheath**- The culm sheath is 15 to 25 cm

long, and gradually tapering with an abruptly ending upper portion. It is deciduous, with an outer surface that is covered by brown hair and a smooth inner surface. The sheaths are covered with white powder and black hair when young. The blade is broadly triangular and heart-shaped. Its apex is somewhat abruptly constricted into an elongated, sharp-pointed tip. The inner surface is hairy and the outer surface smooth. **Flowering**- is gregarious and the flowering cycle is from 30-60 years. Flowers are green. **Fruit**- Fruit is like wheat grain covered with glumes. One kilogram of fruit contains about 14000 seeds. Seed is viable upto 35 days. **Uses** – Traditionally favoured for basketry and woven applications, it is also used for structural purposes. Being a strong bamboo, it lends itself easily to mechanised processing and can be used for making bamboo boards and composites.




(Adapted from http://www.modernistgardening.info/2011_03_01_archive.html)

Figure1.1-Distribution of Bambusa species in Asia-Pacific region



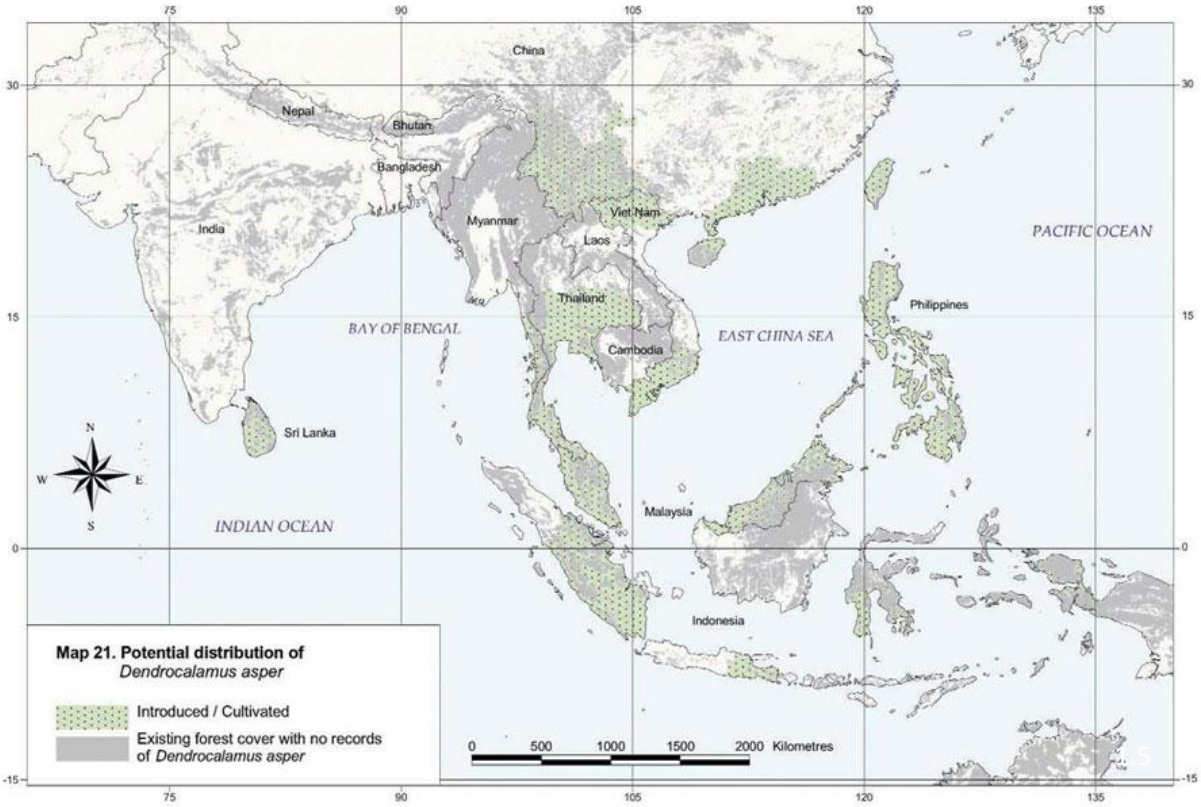

(Adapted from http://www.blueplanetbiomes.org/bengal_bamboo.htm; http://www.bambus-lexikon.de/bambusa-tulda.html; http://tpbg.webfuture.com.tw/plants/plants_1_2_en.php?kind=Poaceae)

Figure1.2- Culms of *B. tulda*; 1.3-Emergence of secondary branch from the culm; 1.4-Leaves

### 1.6.2 *Dendrocalamus asper* Backer Ex K.Heyne

It is commonly known as Giant bamboo, Betung bamboo.

**Distribution:** It is native to Malaysia and Indonesia and was introduced all over South-East Asia (Figure 1.5). **Habitat:** *D. asper* occurs from lowlands up to 1,500m altitude, but thrives well at altitudes of 400–500m in areas with an average annual rainfall of about 2,400mm. *Dendrocalamus asper* grows well on various soil types, even on sandy and rather acidic soils but prefers well-drained heavy soils. **Culm:** Culm has an average height of 20-30m and average diameter of 8-20cm with internodal length of 20-45 cm. Lower nodes are covered with a circle of rootlets. Culms are relatively thick walled, pale green in colour and covered with short hair (Figure 1.6). Buds or branches are present on lower quarter of culm. **Culm sheath:** Culm sheaths are 40–50 cm long and covered with short dark brown hairs which are shed at maturity. The auricles (ear-like parts at the base of the culm sheath) are 7mm high with 5mm long hair on the culm sheath shoulders. The tip of culm sheath is fringed and 7–10mm high. The blade is narrower than the sheath and tapers to a point. It is 25cm long and 35mm wide. **Leaf:** Leaf-blades are lance-shaped and 15–30cm long and 10–25mm wide. Their base is wedge-shaped with a brief stalk-like connection to sheath. Surface of the leaf blade is smooth and hairless (Figure 1.8). **Flowering:** Gregarious. **Uses:** It is a structural timber used for heavy construction in rural communities. It is also used for making boards, furniture, musical instruments, containers, chopsticks, house hold utensils and handicrafts. The young shoots are sweet and hence, considered as a delicious vegetable.




(Adapted http://www.guaduabamboo.com/dendrocalamus-asper.html)

Figure1.5 -Distribution of *Dendrocalamus asper*

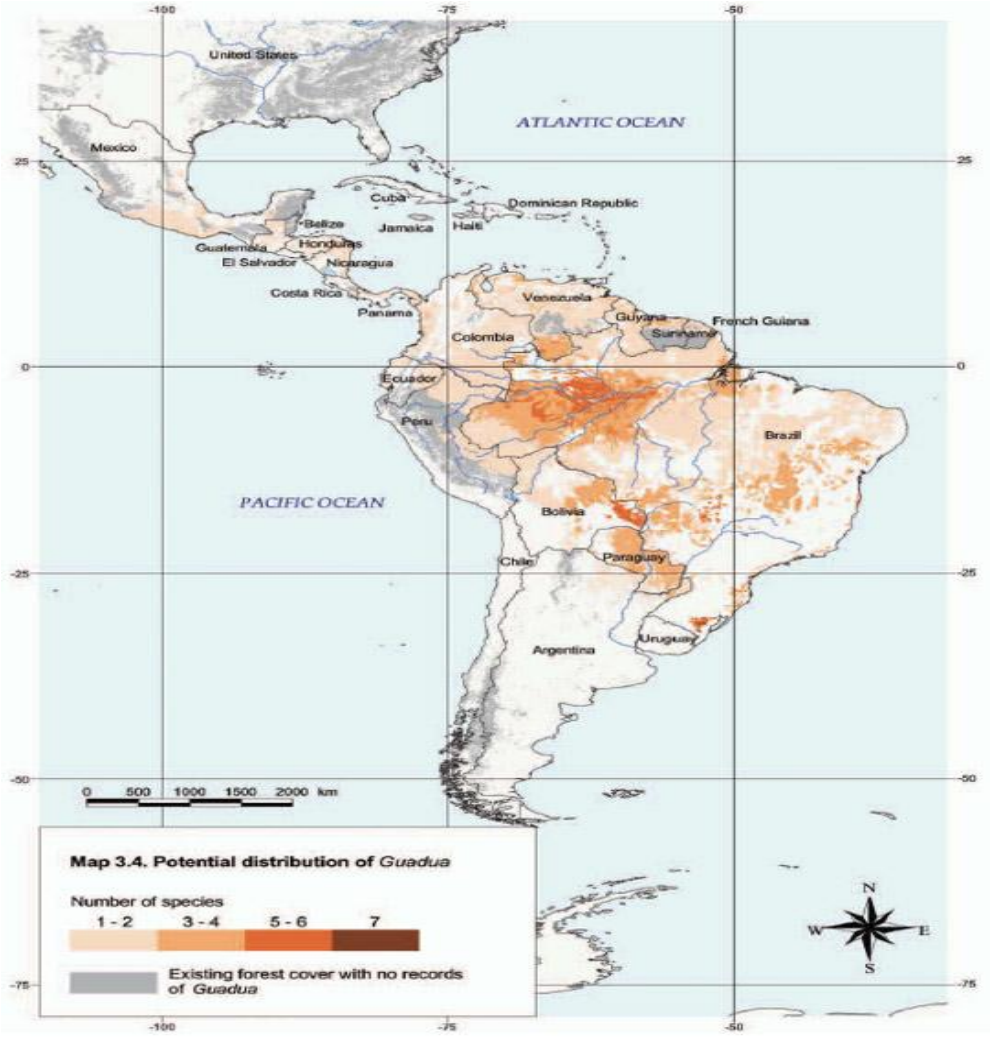

(Adapted from http://www.baanboo.net/plants/; http://www.earthcare.com.au/slides/asper.html; http://www.aryavebiotech.com/bamboo.html)

Figure 1.6- Culms of *D. asper*; 1.7-Emergence of young shoot; 1.8- Leaves

### 1.6.3 *Guadua angustifolia* Kunth

**Distribution**: The *Guadua* is native to Colombia, Ecuador and Venezuela where it forms dominant colonies known as “guaduales”, mainly concentrated in the Andean

region between 0 and 2,000 m above sea level (Figure 1.9). It has been introduced to several Central American and Caribbean countries and even to Asia, North America and Europe. **Habitat**: In Columbia, *Guadua* reaches its optimum development in the central region of the Andes between 500 and 1500 m above sea level at the temperature between 17 and $26^0$C, rainfall of 1200-2500mm/year and a relative humidity of 80-90%. **Culm**: Culm height is 20-30 m and the maximum diameter reported is 25 cm with an average between 9 and 13 cm (Figure 1.10). Culms are woody with root thorns arising from the nodes. Culm internodes are cylindrical, thin walled and about 20 cm long. Lateral branches are tree like with one dominant branch. Lateral branches bear thorns on their internodes (Figure 1.11). **Culm Sheath**: Covered with short, dark brown hair without auricles, culm sheath blade is triangular, erect and covered with tiny hair **Leaf**: Leaf-blades are lance-shaped or oblong, going from a rounded base towards apex. They are 10-20 cm long, 6-12 mm wide and have no cross veins (Figure 1.12). Base of leaf-blade has a brief stalk-like connection to sheath. The stalk is smooth and has no hair. **Rhizome**: Rhizome is of pachymorphic kind with a pattern of sympodial ramification. **Flowering**: Sporadic flowering with peculiarity that the plant does not die after flowering and flourishes annually which is generally associated with hot summers. **Uses**: *Guadua* has been used for numerous applications in all countries of South America . All bamboo houses of South America are built exclusively of *Guadua* and the construction industry in Colombia is the country's largest consumer of *Guadua* poles consuming 70% of this bamboo (Gutierrez, 2000). In Colombia, bridges, hillside houses, kitchen utensils, household objects, fences, stairways and drainage pipes are made of *Guadua.* It also helps in controlling soil erosion.




(Adapted from http://www.guaduabamboo.com/guadua-angustifolia.html)

Figure 1.9- Distribution of *Guadua* species in American continent



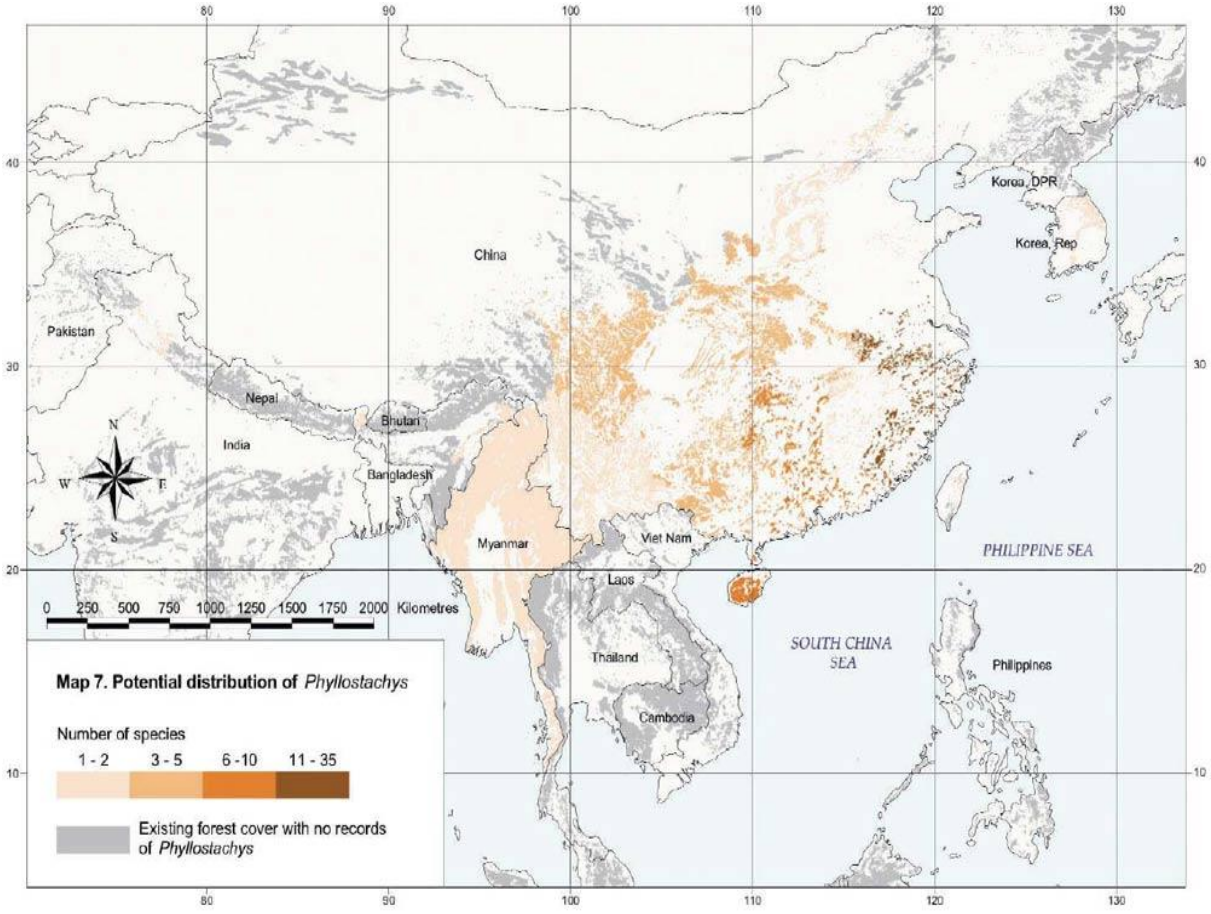

(Adapted from http://www.flickr.com/photos/chdeff_photos/5957596896/; http://www.flickriver.com/photos/72793939@N00/4258323208/)

Figure1.10- Mature culms of *Guadua angustifolia*; 1.11-Thorns on the nodes of secondary branch; 1.12-Tertiary branch bearing leaves

### 1.6.4 *Phyllostachys pubescens* Mazel ex H. De Lehale

It is commonly known as Moso bamboo.

**Distribution**: It is native to mainland China, south of the Yangzie river and distributed mainly in the provinces of Zehchian, Fukien, Chiansh and Hunan and has been naturalized in the neighbouring countries (Figure 1.13). It was introduced to Japan in 1746 and it escaped from the planted areas and expanded by invading native vegetation. **Habitat**: The natural habitat extends approx. 23°30'--32°20' N and 104°30'--122° E. It grows at elevations between 10-1700 m above sea level. Moso belongs to subtropical climate. The mean annual temperature varies from15 to 21° C, with mean temperature of the coldest month being 1 to 12° C and that of the warmest month 26 to 29° C. Moso bamboo can withstand - 18 to - 21 °C in the winter. **Culm**: Culms are over 20 m tall and internodes at midculm reach upto 40 cm in diameter. Annulus of the culm is indistinct (Figures 1.14, 1.15). **Culm sheath**: Sheath scars are prominent. Sheath is thickly covered with brown hair and dark brown specks. Auricles of the sheath are short with developed humeral hair. Sheath ligule is short and broad, bow-shaped with both sides drooping. Blade is green, long triangular or lanceolate. **Leaves**: Leaves are narrow and small, 4-11 cm long and 0.5-1.2 cm wide (Figure 1.16). **Rhizome**: It is leptomorph type. **Flowering**: It occurs after 67 years. **Uses**: It is the most important bamboo species cultivated for culm and shoot production. The shoot is delicious, eaten fresh or processed as canned food or dried food. The culms are used for heavy construction, handicrafts and paper making. The leaves are used in the treatment of arthritic inflammations, hypertension, arteriosclerosis, cardiovascular disease and the sheath of the stem is used in the treatment of nausea and sour stomach.




(Adapted from http://www.guaduabamboo.com/phyllostachys.html)

Figure 1.13- Distribution of *Phyllostachys* species


(Adapted from http://www.scottishbamboo.com/Phyllostachys_Heterocycla_Pubescens_(Moso___Edulis).html; http://www.hotfrog.fr/Entreprises/ethnoplants-plantes-et-graines-rares-atypiques-insolites/Bambou-moso-graines-de-Phyllostachys-pubescens-bambou-exotique-75230; http://www.magicgardenseeds.com/PHY04)

Figure 1.14-Mature culms of *Phyllostachys pubescens*; 1.15-Emergence of young shoots; 1.16-leaves

## 1.7 Rationale and objectives of the thesis

In the last two decades, population growth and new bamboo processing opportunities have led to the over exploitation of bamboo resources, their stricter regulation and even harvesting bans in some countries notwithstanding. Due to the diminishing wood supply, bamboos are now in high demand as raw material sources for furniture, handicraft and many products. Moreover, with the diversification derive by farmers, food security is getting threatened and keeping in mind the virtues of bamboo shoots as food, bamboos provide an alternative source to feed the masses. Because of this, bamboos are over cut by improper harvesting methods, causing serious genetic erosion due to unabated pressure. Hence there is an urgent need for *in situ* and *ex situ* conservation.

The propagation of bamboos is done with seeds, clump divisions, rhizome and culm cuttings. However, gregarious flowering, low seed viability, high costs, problems facing long distance transportation of vegetative propagules and poor efficiency of plant production, compelled development of alternative propagation methods. *In vitro* propagation constitutes a feasible alternative to mass propagate elite bamboos.

Although during the last two decades, considerable efforts have been made to develop *in vitro* methods for propagation for bamboos, yet the gains accrued from this technology at the field level can be seen only in selected species. In most bamboo species, either the micropropagation technique could not be developed or has remained confined to the laboratories without getting commercialized due to constraints encountered at culture initiation and induction of rooting. Keeping in view the importance of bamboos, the present study has been conducted with the following objectives:

1) To develop and standardize mass propagation of *Bambusa tulda*, *Dendrocalamus asper*, *Guadua angustifolia*, *Phyllostachys pubescens* under *in vitro* conditions.

2) To check the clonal fidelity of tissue culture raised plants by RAPD and to study their field performance.

# Chapter 2
# Review of Literature

# 2 Review of literature

## 2.1 Micropropagation of bamboos

Bamboos are giant grasses belonging to family Poaceae with woody culms growing uprightly. Bamboos assume a greater significance in the Indian context because after China, India has the second largest bamboo genetic resources in the world (23 genera and 125 species). Besides being used for meeting the domestic needs, many outstanding industrial products such as quality paper, floor tiles, boards, handicrafts, waxes, incense sticks, foot rulers, matchsticks and activated carbon are made out of bamboos. This versatile and energy efficient natural resource with carbon sequestering efficiency has ability to be used for fuel, fodder and environment management. Bamboo has now emerged as an important replacement of wood for constructional purposes as bamboo housing is gaining popularity, especially in areas prone to disturbance by earthquakes. In the recent years, bamboos have gained importance in social forestry programmes due to their short rotation cycles, fast growth and the possibility of its being harvested progressively on a sustainable basis (Godbole et al. 2002). However, extensive anthropogenic pressures on its local habitats have led to dwindling of natural stands and their regeneration. The immense utility and versatility of this natural resource has led to its over-exploitation. The traditional method of vegetative propagation fails to meet the ever increasing demand for industrial and commercial applications. Conventional methods like clump division, offsets, rhizomes and culm cuttings suffer from serious drawbacks (Banik, 1987). Slow multiplication of offsets, unpredictable flowering cycles, plant death after gregarious flowering, poor seed set and viability and remembrance of age makes conservation of bamboo germplasm imperative. Micropropagation appears to be the feasible alternative in this regard by which healthy, disease free and genetically

uniform planting material can be obtained. Moreover, the cultures can be maintained in juvenile phase for long durations.

The present review is intended to consolidate significant advances made to date in the propagation of bamboos by *in vitro* methods and to provide an insight for carrying out future work as well.

Although initial reports on regeneration of bamboo plantlets through embryo culture appeared in the late sixties (Alexander and Rao, 1968), but a complete protocol for micropropagation of *Bambusa bambos* (=*Bambusa arundinacea*) through somatic embryogenesis using seeds (caryopsis) was first described by Mehta et al. (1982).

Nadgir et al. (1984) employed forced axillary branching for the multiplication of shoots derived from seedlings of *Dendrocalamus strictus* on a complex liquid MS medium supplemented with cytokinins and coconut milk by using rotary shakers. Later on, Dekkers and Rao (1989) also obtained multiple shoots in *D. strictus* but with poor root induction. Similarly, multiple shoots were obtained by using seedling cultures of *D. strictus* by Kumar (1994) which could not be continued for long due to lack of meristematic tissue at the base of the explants. Since then, micropropagation methods 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, low rooting frequency etc. However, multiplication of superior bamboo clumps with desirable traits is possible only with the use of field selected elite adult plant tissues than with seed/seedling explants.

For micropropagation of bamboos, all the three techniques of *in vitro* propagation *viz* forced axillary branching, somatic embryogenesis and *de novo* adventitious shoot

formation have been exploited. Forced axillary branching is, however, considered to be the best since it ensures the genetic stability of the clones.

In the recent years, extensive research regarding micropropagation of bamboos has been done (Paranjothy *et al.*1990, Saxena, 1990; Sood et al. 1992; Ramanayake et al.1997; Bag et al. 2000; Arya et al. 2002; Das et al. 2005a; Agnihotri et al. 2009). Successful tissue culture protocols have been explored and some of the pioneering research outcomes are being reviewed in the following pages:

**2.2 Stages involved in micropropagation:**

In micropropagation, pre-existing meristems are induced to proliferate on nutrient enriched medium in the presence of growth adjuvants and the protocol involves the following sequential stages: I) initiation of aseptic cultures II) shoot proliferation III) rooting of microshoots and IV) hardening and field transfer of plantlets.

**2.2.1 Initiation of aseptic cultures**

**2.2.1.1 Explant**

For carrying out *in vitro* propagation, different explants have been employed by different workers but nodal explants and seeds are the most commonly used ones (John et al. 1997). The use of starting material and technique for propagation are crucial factors for bamboo propagation (Gielis, 1999). Major advantage of using seeds is that they establish a new generation and the technology is comparatively easier. After the first report of micropropagation in *Dendrocalamus strictus* using seeds by Alexander and Rao (1968), many authors have successfully established the micropropagation protocols in different bamboo species using seeds/seedlings explants like *D. strictus* (Nadgir et al. 1984; Rao et al 1985; Dekkers and Rao 1989; Preetha et al. 1992; Shirgurkar et al. 1996; Maity and Ghosh, 1997; Reddy, 2006 ; Ravi kumar et al. 1998; Singh et al. 2000), *Bambusa tulda* (Saxena, 1990),

*Dendrocalamus hamiltonii* (Chambers et al. 1991), *Bambusa bambos* (Kapoor and Rao, 2006), *D. asper* (Arya et al. 1999), *Bambusa vulgaris* (Rout and Das, 1994), *Bambusa nutans* (Yashodha et al. 1997). However, 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).

The success with explants taken from mature plants of bamboo for propagation has been limited to a few cases only. Multiplication from adult culms is restricted by many factors (Lin and Chang, 1998) and maturation of the tree species adversely affects the morphogenetic potential of the axillary buds (Pierik, 1990). The impediments associated with the use of adult tissue are 1) endogenous contamination 2) hyperhydricity and instability in multiplication rates 3) difficulty in inducing rooting and 4) higher phenolic exudations. Despite these constraints, 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; Mudoi et al. 2009), *Bambusa edulis* (Lin et al. 1998), *B. glaucescens* (Banik, 1987; Shirin and Rana, 2007), *Bambusa vulgaris* (Nadgir et al. 1984; Gielis, 1999; Ramanayake et al. 2006; Ndiaya et al. 2006), *Bambusa tulda* (Das and Pal, 2005b; Mishra et al. 2007), *Dendrocalamus giganteus* (Ramanayake et al. 1997), *Dendrocalamus hamiltonii* (Sood et al. 2002a,b; Agnihotri and Nandi, 2009; Agnihotri, 2009), *Dendrocalamus strictus* (Nadgir, 1984; Chaturvedi et al, 1993; Ravikumar, 1998). Some researchers have also used inflorescence explants for establishing protocols for multiple shoot proliferation in

bamboos like *Bambusa edulis* (Lin et al. 2005a), *Dendrocalamus asper* (Arya et al. 2008a), *Dendrocalamus giganteus* (Ramanayake, 1998), *Dendrocalamus latiflorus* (Lin et al. 2006).

#### 2.2.1.2 Surface sterilization

Surface sterilization of seeds usually involves the treatment of dehusked seeds with 70% ethanol (v/v) for 20-30 seconds followed by treatment with $HgCl_2$ (0.05-0.1%) for 15-20 minutes and then repeated washings with sterilized distilled water before inoculation (Nadgauda *et al.* 1997a; Bag *et al.* 2000). In *Dendrocalamus hamiltonii*, Chambers et al. (1991) disinfected seeds in 20% Domestos containing 4% sodium hypochloride for 30 minutes followed by rinsing in sterile distilled water. Sodium hypochloride (4%) was also employed by Arya et al. (1999) for surface sterilization of *Dendrocalamus asper* seeds.

Bacterial and fungal contamination in tissue culture is well documented (Paranjothy et al, 1990; Saxena, 1990; Ramanayake and Yakandewala, 1997; Reed et al. 1998) and the failure of surface sterilization procedures to produce aseptic cultures is a major problem with woody plants (Phillips et al. 1994a). Different experimental procedures including chemical sterilization and antibiotics have been used at various levels of success to minimize or eliminate such contamination. However, the type, concentration and duration of treatment vary for different plant tissue cultures. Therefore, it is pertinent to optimize the treatment strategy before its use (Buckley et al. 1995).

In bamboos, the nodal explants from mature culms require more severe surface sterilization treatments than seeds due to the persistent microbial contamination. Different strategy has been employed by different workers to counter microbial contamination. Ramanayake and Yakandawala (1997), treated nodal explants of *D.*

*giganteus* with saturated solution of bleaching powder for 10 minutes followed by treatment with Benlate for 1 hour. They were surface sterilized again in a 0.3% solution of $HgCl_2$ for 10 minutes and rinsed in sterile water before inoculating on MS medium. In *D. hamiltonii*, Sood et al. (1992) performed presterilization of nodal cuttings with 1% solution of calcium hypochloride for 10 minutes, followed by a wash in streptomycin sulphate and quik dip in 90% alcohol. These were further treated with 0.04 % $HgCl_2$ for 6 minutes followed by repeated washings in distilled water.

While in *Guadua angustifolia*, pretreatments prior to disinfection included immersion in an alkaline solution of Extran for 10 minutes and a combination of the bactericide Agri-mycin and the fungicide Benomyl at the concentration of 2g/l each for 10 minutes (Jimenez et al. 2006). In *Bambusa oldhami*, Lin et al. (2007a) soaked excised nodes in 2% sodium hypochloride with sonication for 30 minutes. Sodium hypochloride was also employed by different researchers for achieving surface sterilization in other bamboo species (Lin and Chang, 1998; Ogita et al. 2008). Thakur and Sood (2006) developed a unique and efficient procedure for sterilization in bamboos with elongated internodes by treating explants with sterilizing agents in a stoppered glass measuring cylinder.

**2.2.1.3 Removal of prophylls**

Hirimburgama and Gamage (1995) reported that removal of prophylls increased the event of bud break in *Bambusa vulgaris***.**

**2.2.1.4 Choice of medium**

For initiation of aseptic cultures, various media were employed by different workers for inducing seed germination and bud break in nodal explants. For the germination of bamboo seeds, White's medium was recommended by some researchers (Alexander and Rao, 1968; Nadgir et al. 1984; Shirgurkar et al. 1996; Nadgauda et

al. 1997b, Ravikumar et al. 1998). In *Dendrocalamus asper*, Arya et al. (1999) used Woody Plant Medium for germination of seeds. But perusal of literature reveals that MS medium is the most widely used medium for inducing bud break in nodal explants (Sood et al. 1992; Chambers et al. 1991; Ramanayake and Yakandawala, 1997; Rout and Das, 1997; Lin and Chang, 1998; Singh et al. 2000; Sanjaya et al. 2005; Das and Pal, 2005; Lin et al. 2007a; Agnihotri et al. 2009; Arya et al. 2008a ;Negi and Saxena, 2011. Ndiaye et al. (2006) tested four basal media viz. MS medium, Gamborg's medium, Lloyd and Crown medium (1980) and modified MS medium for evaluating regeneration efficiency in *B. vulgaris* and observed that modified MS medium showed highest rate of regeneration of 100%. Even with MS medium different strengths were employed by many researchers for inducing bud break. Sood et al. (1992) observed sprouting in nodal explants of *D. hamiltonii* on half strength MS medium withih 10 days. Similarly, Godbole et al. (2002) also induced sprouting on half strength MS medium in *D. hamiltonii.*

**2.2.1.5 Effect of season on bud break**

McClure (1966) observed that in nature, dormancy and breaking of dormancy in buds of bamboo varied with their position on the plant, the season of the year and the species. In *D. giganteus*, the midculm nodes of secondary branches have been reported to be the best explants for axillary shoot initiation (Ramanayake and Yakandawala, 1997). Saxena and Bhojwani (1993) found that *in vitro* bud-break in *D. longispathus* took place during the monsoon. In tropical species including bamboo changes in the environment such as those caused by rainfall or its onset may trigger the synthesis or breakdown of endogenous substances that control growth responses. The inverse relationship between *in vitro* bud-break and systemic culture contaminants in *D. giganteus* (Ramanayake and Yakandawala, 1997) and in *B.*

*vulgaris* (Kumari and Ramanayake, 1996) indicates that these substances could also control the growth of microflora harboured within the tissues. Earlier in *D.strictus* too, culture initiation of mature field grown culms was difficult due to contaminants (Nadgir et al. 1984). In *B. nutans*, Mehta et al. (2010) concluded that best months for establishing aseptic cultures were February and December when low contamination rates were obtained. While Negi and Saxena, (2011) reported that 100% bud break was achieved in explants when the cultures were initiated in the month of July.

**2.2.1.6 Position of node on the culm**

Initiation of aseptic cultures is also influenced by the position of node on the culm.

In *B. vulgaris,* Hirimburgama and Gamage (1995) observed that middle nodes responded better than the basal or terminal buds. Saxena and Bhojwani (1993) reported that in *Dendrocalamus longispathus* midculm nodes of secondary branches proved to be the best explants for axillary shoot initiation. While in *G. angustifolia,* cultures were initiated using nodal segments of lower healthy lateral branches (Jimenez et al. 2006).

**2.2.2 Shoot multiplication in nodal explant**

Nadgir et al. (1984) successfully multiplied shoots derived from nodal explants from the adult plants of *Bambusa bambos, B. vulgaris* and *Dendrocalamus strictus*. Although, Chaturvedi et al. (1993) failed to achieve multiplication of shoots in *D. strictus* but succeeded in inducing rooting (30%) by placing the nodal segments upside down on a complex medium containing 2,4-D and phloroglucinol. Later, Banik (1987) reported axillary shoot proliferation on medium supplemented with BAP (5µM), NAA (5.37 µM) and activated charcoal (3g/l) but could not sustain further multiplication of these shoots. Saxena and Bhojwani (1993) established a

micropropagation protocol through forced axillary branching in *Dendrocalamus longispathus* on MS medium supplemented with BAP and Kn.

Ramanayake and Yakandwala (1997) micropropagated giant bamboo (*Dendrocalamus giganteus*) and found an inverse relationship between bud-break and contamination. Incorporation of benlate (1g/l) into the medium reduced culture contaminants significantly while coumarin (68.42 µM) along with IBA (14.76 µM) prevented browning of propagules and induced new shoots and roots. Ravikumar et al. (1998) attempted *in vitro* shoot propagation of *D. strictus* using both seedlings and axillary buds of 10 year old plants and found that the seedlings performed better than mature explants on a medium containing BAP (2.22 µM), Kn (2.32 µM) and coconut water (200ml/l). The frequency of shoot formation was much less in case of mature explants. Arya et al. (1998) have reported axillary shoot proliferation using young and juvenile shoots from nursery raised three year old plants of *B. bambos* and emphasized the importance of growth regulator's concentration in controlling the morphogenetic events. Proliferation of healthy shoots was observed at lower doses of BAP (4.44-22.2 µM) while higher doses of BAP (6-10 µM) induced thin, leaf like shoots.

Arya et al. (2002) have described the micropropagation protocol for *Dendrocalamus asper* using nodal shoots and seed culture and found presence of BAP (0.44-66.6 µM) to be essential for axillary bud activation. In case of *Bambusa edulis*, TDZ was found to be effective for multiple shoot proliferation from nodal explant as reported by Lin et al. (1998) but found that the concentration of TDZ in the medium determined the response: higher concentration (6µM) inhibited elongation of shoots and led to considerable vitrification and albino shoot production.

In yet another study using *Thamnocalamus spathiflorus*, Bag et al. (2000) accomplished multiple shoot formation from nodal explants in the presence of BAP (5 µM) and IBA (1 µM). Initially very little response was observed on multiplication media but response picked up after a few sub-culturings. Sood et al. (2002a) have described an efficient protocol for *in vitro* propagation of *D. hamiltonii* using explants from 3-year old field selected seedlings. Recently, in the same species, Agnihotri et al. (2009) have obtained 20-fold multiplication on BAP (8µM) and NAA (1µM).

Sanjaya et al. (2005) obtained high frequency multiple shoot induction in *Pseudoxytenanthera stocksii* Munro on NAA (2.68 µM) and BAP (4.40 µM). MS liquid medium fortified with NAA (2.60 µM), BAP (4.40 µM ) and additives: ascorbic acid (283.93 µM), citric acid (118.10 µM), cysteine (104.04 µM) and glutamine (342.24 µM) supported further multiplication. Ramanayake et al. (2006) reported continuous shoot proliferation of nodal explants from adult field culms of *Bambusa vulgaris* on BAP supplemented MS medium. Effect of BAP in promoting shoot regeneration was highlighted by Ndiaye et al. (2006) in *B. vulgaris*. In *Guadua angustifolia,* an increase in concentration of BAP directly influenced the production of lateral shoots (Jimenez et al. 2006).

In an interesting report, Mishra et al. (2007) found that liquid medium enriched with glutamine, IAA and BAP supported maximum shoot multiplication rate of two-fold in *B. tulda.* A year later, Lin et al. (2007a) obtained mosaic virus-free plantlets of *B. oldhami* and TDZ was found to be a stable and effective cytokinin for proliferation in long-term subcultures. Arya et al. (2008) have reported direct shoot regeneration from immature inflorescence explants in *D. asper* in the presence of BAP (31.08 µM). Recently, Mudoi et al. (2009) have been successful in obtaining ten-fold continuous shoot proliferation in *B. balcooa* on BAP (4.44 µM) fortified medium.

### 2.2.2.1 Multiplication medium

Several workers have reported higher rates of shoot multiplication and improved growth in liquid medium (Nadgauda et al. 1990; Sood et al. 1992; Ramanayake and Yakandawala, 1997; Ravi kumar et al. 1998; Singh et al. 2001;Das and Pal 2005a; Sanjaya et al. 2005; Jimenez et al. 2006; Mishra et al. 2007; Negi and Saxena, 2011). Nadgir (1984) multiplied shoots in liquid medium and maintained the cultures on a shaker for entire shoot multiplication cycle of 6-7 weeks. In *D. giganteus*, liquid cultures in shaker responded faster to axillary shoot proliferation than stationary cultures (Ramanayake and Yakandawala, 1997).

The poor performance in solid medium can be attributed to the binding of water and absorption of minerals and growth hormones by gelling agents, resulting in a restricted supply of the former to the growing shoots (Debergh, 1983). In comparison, liquid medium facilitates the easier uptake and better absorption of medium components as the growing shoots are in direct contact with the medium (Debergh, 1983). Moreover, it is economical if shakers are not employed. Saxena (1990) also obtained the highest shoot multiplication rates and growth in liquid medium in *B. tulda.* In addition, shoots were greener and healthier in liquid medium than in semi-solid medium; lamina of leaf which was almost inconspicuous or under developed on agar medium was green and well developed in the liquid medium.

However, a continuous culture in liquid medium may cause vitrification of shoots (Saxena and Bhojwani, 1993). Ramanayake and Yakandwala (1997) used luffa pieces to support shoots while Saxena and Bhojwani (1993) controlled vitrification of shoots by supporting them on pieces of foam. Negi and Saxena (2011) avoided the problem of hyperhydration by supporting shoot clusters by shredded Whatman filter paper strips.

During *in vitro* propagation studies in bamboos, agar appeared to be the most widely used gelling agent (Shirgurkar et al. 1996; Arya et al. 1999; Godbole et al. 2002; Kapoor and Rao, 2006; Lin et al. 2007a; Arya et al. 2008a,b; Mudoi et al. 2009). But gelling agents other than agar have also been used by different researchers. Bag et al (2000) recommended the use of medium gelled with phytagel for comparable multiplication efficiency. Similarly, Ramanayake and Yakandwala (1997), Zamora et al. (1988), Agnihotri et al. (2009) recommended the use of phytagel for healthy growth of plants. There are some reports depicting the use of gelrite as a solidifying agent in bamboo micropropagation studies (Lin et al. 1998; Ndiaye et al. 2006; Negi and Saxena, 2011), whereas in *D. hamiltonii,* bacto-agar (Difco) was used by Chambers et al. (1991).

**2.2.2.2 Effect of higher concentration of cytokinins**

In bamboos, the use of higher concentrations of BAP have been found to induce negative effect on shoot length (Shirin and Arya, 2003; Shirin and Rana, 2007). Prutpongse and Gavinlertvatana (1992) highlighted that shoot lengths of *D. asper* genotypes decreased as BAP concentration was increased from 13.2 to 22 µM. Yashodha et al. (1997) also noticed the formation of stunted shoots in *B. nutans* and *D. membranaceus* with higher doses of BAP. Vongvijitra (1988) reported inhibition of rooting with use of higher concentration of BAP during shoot formation. Godbole et al. (2003) reported that at higher concentration of BAP, the leaves remained curled up and failed to expand in *D. hamiltonii*. Arya and Sharma (1998) had similar observations that higher doses of BAP induced thin leafy shoots. Negi and Saxena (2011) also reported similar findings that higher concentration of BAP inhibited the shoot proliferation in *B. nutans.*

### 2.2.2.3 Synergistic effect of cytokinins

Use of the cytokinin BAP with Kn in the basal medium has resulted in higher rate of shoot multiplication in several species of bamboos. In *B. glaucescens*, Shirin and Rana (2007) observed a synergism between two cytokinins and the best interaction providing the highest rate of shoot multiplication (4 fold) was a combination of BAP (5 µM) and Kn (15 µM). Even in earlier findings, positive effects of BAP and Kn interactions on multiplication of bamboo shoots have been noted. Nadgir et al. (1984) have reported the requirements of higher concentration of BAP and also an additional supplementation of Kn for explants taken from mature culms of 3 species of bamboo- *D. strictus, B. arundinacea* and *B. vulgaris.* Similarly, Saxena (1990) and Das and Pal (2005a) also recommended the use of Kn along with BAP in the medium in order to get an enhanced rate of shoot multiplication through forced axillary branching in *B. tulda* and *B. balcooa* respectively. A synergistic effect of BAP (15 µM) and Kn (15 µM) combination was also reported by Shirin et al. (2003) which resulted in a higher rate of shoot multiplication in *B. vulgaris* from axillary buds of mature culms.

### 2.2.2.4 Use of additional growth adjuvants

Use of coconut milk for enhanced proliferation of culures has been advocated by many workers (Nadgir et al. 1984). Saxena and Bhojwani (1993) observed an enhanced rate of shoot multiplication in *D. longispathus* with the use of coconut milk (10%). Nadgauda et al. (1990) induced shoot proliferation in seedling derived cultures of *B. arundinacea* and *D. brandisii* in medium supplemented with coconut milk. Ramanayake and Yakandawala (1997) observed that in the absence of coconut milk, a significantly lower number of shoots were obtained in *D. giganteus.*

### 2.2.2.5 Carbon source

Sucrose is the most preferred carbon source for *in vitro* studies in bamboos. But the concentration of sucrose employed for multiplication of cultures varied with the bamboo species. Use of 3% sucrose has been recommended for shoot proliferation in many bamboo species viz *D. giganteus* (Ramanayake and Yakandawala, 1997), *D. hamiltonii* (Sood et al. 1992), *D. asper* (Arya et al. 1999), *G. angustifolia* (Jimenez et al. 2006), *B .oldhamii* (Lin et al. 2007a), *B. tulda* (Mishra et al. 2007), *B. vulgaris* (Ndiaye et al. 2006), *B. glaucescens* (Shirin and Rana, 2007). Addition of 2% sucrose has also been advocated in many bamboos like *B. arundinacea* (Nadgir et al. 1984), *B. tulda* (Saxena, 1990), *B. longispathus* (Saxena and Bhojwani, 1993), *D. hamiltonii* (Agnohotri et al. 2009) and *D. strictus* (Nadgir et al. 1984; Shirgurkar et al. 1996; Ravikumar et al. 1998). Saxena (1990) reported that incorporation of higher levels of sucrose during micropropagation of *B. tulda* caused severe albinism, while in *B. bambos,* an increase in sucrose concentration was found to result in more rhizome formation (Kapoor and Rao, 2006).

### 2.2.2.6 Single shoot vs shoot clump

Shoot clumps rather than single shoots were found to be effective for multiplication of bamboo plants (Arya et al.1999; Ramanayake et al. 2001, Sood et al. 1992). Ravikumar et al. (1998) reported that for further sustained growth and multiplication in *D. strictus* , the shoots should be transferred in groups of 5-7 during subculturing. Bag et al. (2000) highlighted that propagules containing a minimum of three to four shoots proliferated at a maximum rate where as single shoots proliferated at a much slower rate. Even in *D. asper,* multiplication rate declined sharply if propagules of less than 3 shoots were cultured (Arya et al. 1999).

### 2.2.3 Rooting

Rooting is the major problem encountered during micropropagation of bamboos. Low rooting friequencies 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. Many factors influence the response of shoots to the rooting media. Chaturvedi et al*.* (1993) noted that explants taken in July - August showed better rooting ability (approximately 30%) than those taken in September - October (approximately 5%). According to them, the metabolic status of the donor plant affected the rooting efficiency of *in vitro* plants. Saxena and Bhojwani (1993) also reported a similar effect of season on rooting efficiency. Moreover, it has been observed that shoot clusters each containing 3-5 shoots responded better for rooting efficiency rather than the single shoot (Sood et al*.* 1992; and Bag et al*.* 2000). In bamboos, rooting efficiency and concentrations and combinations of auxins required for inducing rooting varied with species. Vongvijitra (1988) found that *D. brandisii* shoots could be rooted in plain culture medium or in the presence of low concentrations of BAP, while *D. membranaceous* required supplementation with IBA or NAA. Incorporation of activated charcoal alone in the medium induced rooting in *D. strictus* (Nadgir et al*.* 1984). In some of the reports additional growth adjuvants apart from auxins were employed for inducing rooting.

Nadgir et al. (1984) successfully multiplied shoots derived from nodal explants of adult *Bambusa bambos, B. vulgaris* and *Dendrocalamus strictus*. However, rooting occurred only in *D. strictus* with 20% efficiency. Although, Chaturvedi et al. (1993) failed to achieve multiplication of shoots in *D. strictus* but succeeded in inducing rooting (30%) by placing the nodal segments upside down on a complex medium containing 2,4-D and phloroglucinol. In *Bambusa tulda* rooting to the tune of 90%

was accomplished on a combination of IAA (10 µM) and coumarin (68 µM) (Saxena, 1990). In *D. hamiltonii* rooting and subsequent rhizome formation was accomplished in the presence of IBA (2.46µM or 4.92µM) or NAA (2.68µM) and 3% sucrose (Sood et al. 1992). Saxena and Bhojwani (1993) established a micropropagation protocol in *Dendrocalamus longispathus* where 73% shoots responded to rooting in the presence of IAA, IBA and coumarin and reported more than 85% survival of the plantlets during hardening. In case of *Bambusa edulis*, addition of 2,4-D along with TDZ helped in root induction (Lin et al. 1998)

In yet another study using *Thamnocalamus spathiflorus* (Bag et al. 2000), an initial exposure to IBA and its subsequent withdrawal from the medium was found to be very effective for overall root development. Sood et al. (2002a) have described an efficient protocol for *in vitro* propagation of *D. hamiltonii* and advocated the use of coumarin or choline chloride for root induction. In the same species, pulse treatment of propagules with IBA for 7days followed by subculturing to IBA- free medium was found to be most effective for root development (Agnihotri et al. 2009).

During micropropagation studies of *D. giganteus*, Ramanayake et al. (2006) emphasized that a pre-exposure of shoots to TDZ (2.27 µM) for 2-3 subcultures enhanced rooting efficiency and continuously illuminated shoots showed 100% rooting as compared to 83% rooting in shoots which were exposed to a 12h photoperiod. Ndiaye et al. (2006) achieved rooting in 45.85% shoots in the presence of IBA (48.42µM) in *B. vulgaris*. In *Guadua angustifolia,* rooting occurred spontaneously in 100 % of the explants that developed lateral shoots (Jimenez et al. 2006).

Mishra et al. (2007) achieved rooting in *B. tulda* with supplementation of coumarin to the liquid medium. Arya et al. (2008) reported that IBA (49.2 µM) was quite effective for root induction with 90-95% frequency in the shoots regenerated from immature inflorescence of *D. asper*. A year later, Mudoi et al. (2009) successfully induced 75% rooting on the combination of BAP (4.44 µM) and NAA (16.11 µM).

### 2.2.4 Acclimatization

Various types of potting mixtures were successfully used by different researches for acclimatization. Most of the reports revealed the use of sand:soil:manure in equal proportion (1:1:1) (Kapoor and Rao, 2006; Sood et al. 2002a; Godbole et al. 2002; Agnihotri et al. 2009). In *B. tulda* , Saxena (1990) transferred the plantlets to soilrite in cap bottles and irrigated them with hormone and sucrose free Quoirin and Lepoivre (1977) medium before transferring them to the potting mix of soil:soilrite:organic manure. Ramanayake and Yakandawala (1997) used coir dust and loam in equal proportions to harden the *D. giganteus* plantlets. Negi and Saxena (2011) tested mixture of soil and agropeat in different ratios and obtained hardening survival of 98% in 2:1 soil:agropeat mixture. While Ndiaye et al. (2006) achieved acclimatization of rooted shoots in pots containing perlite and peat in the ratio of 1:2.
In *Pseudoxytenanthera stocksii,* established plants were transferred to potting mixture of compost 50%:sand 40%:soil10% and incubated in a mist chamber for 3 weeks (Sanjaya et al. 2005).

## 2.3 Molecular characterization

Plant tissue culture is regarded as a major area of biotechnology because of its potentiality to regenerate elite genetic resources but scaling up of any micropropagation protocol is severely hindered due to incidences of somaclonal variations (Larkin and Scowcroft, 1981). Somaclonal variations are induced due to

the stress imposed on the plant during propagation and is incorporated in the form of DNA methylation, chromosome rearrangements and point mutations (Phillips et al., 1994b). The occurrence of somaclonal variations is a potential drawback when large scale propagation of an elite plant species is intended. Here the clonal fidelity is essential to maintain the advantages of desired elite genotype (Rahman and Rajora, 2001).

Among the various methods of *in vitro* propagation, the axillary shoot proliferation is a least susceptible to genetic modification (Shenoy and Vasil, 1992). However, the possibility of somaclonal variations cannot be ruled out even with this method as reported in *Populus deltoids* (Rani et al. 1995), *Robinia pseudoacacia* (Bindiya and Kanwar, 2003), *Hagenia abyssinica* (Feyissa et al. 2007), *Olea europaea* (Peyvandi et al. 2009). Several techniques are available to assess tissue culture induced variations in plants such as morphological discriptions, physiological supervisions, cytological studies, isozymes (Gupta and Varshney, 1999) and molecular markers. However, the molecular markers are regarded as rapid, sensitive and more reliable alternative approach (Sharma et al. 2008a; Sharma et al. 2008b). 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).

There are very few reports which can confirm the clonal fidelity of bamboo plantlets derived from axillary bud proliferation. The scarcity of reports on ascertaining the genetic fidelity of tissue culture raised plantlets can jeopardise the quality of micropropogated plants, especially in perennials like bamboo 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.

Earlier, Das and Pal (2005a) established the clonal fidelity of regenerants of *Bambusa tulda* and *B. balcooa* using only four markers to assess their genetic uniformity among the regenerants. Later, Negi and Saxena (2010) employed 15 ISSR markers to validate the clonal fidelity of *in vitro* raised *B. balcooa* plantlets through the axillary bud proliferation. Recently, Nadha et al. (2011) employed 15 RAPD and 17 ISSR markers to assess genetic stability in the *in vitro* raised clones of *G. angustifolia.* No polymorphism was observed, thus confirming true to type nature of *Guadua* clones.

**2.4 Somatic embryogenesis**

Somatic embryogenesis in bamboos has been reported by many workers. It is desirable to develop methods based on somatic embryogenesis to reduce costs of bamboo liners and planting materials for forestry. Somatic embryogenesis in bamboo was reported for the first time by Mehta et al. (1982) in *B. arundinacea*. Thereafter, successful reports are available in many species of bamboos.

Induction of embryogenic calli have been reported from different explants like leaves in *B. multiplex* (Jullien and Van, 1994), anthers in *Sinocalamus latiflorus* (Tsay et al. 1990), from roots of *B. beecheyana* Munro var . beecheyana (Chang and Lan, 1995), from nodal segment in *B. vulgaris* (Rout and Das, 1997; Gielis, 1999) and *B. ventricosa* (Gielis, 1999) and from inflorescences of *B. oldhamii* Munro (Yeh and Chang, 1986a), *B. beecheyana* Munro var. beecheyana (Yeh and Chang, 1986b), *B. edulis* (Lin et al. 2004a). However, in these cases regeneration of embryos was either not achieved or occurred at too low a frequency to be used on a commercial scale.

In 1985, Rao et al. were able to induce somatic embryogenesis in *D. strictus* by culturing seeds on $B_5$ basal medium supplemented with 2,4-D. About 40% of embryoids developed into plantlets when they were transferred to a germination medium containing IBA and NAA. Woods et al. (1992) developed an efficient protocol for obtaining somatic embryos in *Otatea acuminata azetecorum* from zygotic embryos. Optimum somatic embryogenesis and plant regeneration were achieved by culturing in dark on MS medium containing 2,4-D and BAP. Rout and Das (1994) reported somatic embryogenesis from zygotic embryos and nodal segments of *in vitro* grown seedlings of *B. vulgaris*, *D. giganteus* and *D. strictus*. Best and sustained callus growth occurred on MS medium supplemented with 2,4-D (13.57 µM) and Kn (1.16 µM). When subcultured to half strength MS medium containing Kn (2.32 µM), adenine sulphate (27.14 µM) and 2,4-D (9.04 µM), calli became embryogenic after 5 weeks. Embryos eventually developed into plantlets after 4 weeks of subculture in all the 3 species.

In 1986(a), Yeh et al. reported an efficient protocol for high frequency of plant regeneration through somatic embryogenesis in *B. oldhamii*. The embryogenic callus was initiated from young inflorescence explants maintained on MS medium supplemented with 2,4-D (13.57 µM), Kn (9.29 µM) and a high content of sucrose (60g/l). Prolonged culture in the embryoid induction medium or transfer of embryogenic callus to auxin free medium resulted in continued development and germination of embryoids. In the same year, Yeh et al. (1986b) reported embryogenic callus in *B. beecheyana* from young florets on modified MS medium supplemented with 2,4-D (13.57 µM), Kn (9.29 µM) and a high content of sucrose (6%). The embryos germinated spontaneously to yield complete plants on this medium with or without hormonal adjuvant. Hassan and Debergh (1987) cultured

leaf explants of *Phyllostachys viridis* on MS medium supplemented with 2,4-D and observed numerous embryoids. On transfer to MS medium lacking hormones, plantlets developed within two weeks and were later successfully transferred to field.

Saxena and Dhawan (1999) used *D. strictus* seeds to obtain embryogenic callus on MS medium supplemented with 2,4-D (30 µM). Shifting of this embryogenic callus to MS medium containing 2,4-D (10 µM) and IBA (20 µM) resulted in 2 to 5 fold multiplication of somatic embryos every 5 weeks. Browning of the medium was the major problem encountered in the experiments. Incorporation of Polyvinylpyrolidone (PVP, 250 mg/l) to the culture medium proved very effective in overcoming this problem. In *D. hamiltonii*, Godbole et al. (2002) used new sprouts from nodal segments of field-selected elite bamboos for inducing embryogenic callus. MS medium supplemented with BAP (4.44 µM) and 2,4-D (4.52 µM) was found to be essential for callusing. Elimination of 2,4-D and a corresponding increase in BAP concentration induced embryogenesis. Starch deposition and amylase accumulation was observed in callus during their study (Godbole et al. 2004). Lin et al. (2003a) was successful in achieving somatic embryogenesis in *B. edulis* from nodal and internodal tissues on MS medium supplemented with Kn (9.2 µM), 2,4-D (13.6 µM), coconut milk (0.1%) and sucrose (6%). Addition of TDZ (0.046 µM) to the MS medium containing 2,4-D (13.6 µM) and 3% sucrose enhanced the proliferation of embryos. In this study, TDZ was also found to be promotive for germination of somatic embryos. Singh et al. (2003) has successfully regenerated NaCl tolerant plantlets of *D. strictus* from NaCl tolerant embryogenic callus via somatic embryogenesis. In their experiment, 39% of mature somatic embryos tolerant to 100 mM NaCl were generated and converted into plantlets in germination medium containing 100mM NaCl.

A reliable protocol for mass propagation via somatic embryogenesis in mature plants of *B. balcooa* has been established using pseudospiklets by Gillis et al. (2007). The explants when incubated on MS medium containing 2,4-D (4.52 µM) in continuous darkness formed embryogenic callus. Shifting of this callus to basal MS medium led to the formation of somatic embryos. In *D. asper* , Arya et al. (2008) successfully achieved somatic embryogenesis from nodal tissues and basal part of leaves. Callus cultures obtained on 2,4-D (30µM) became highly embryogenic when transferred on to medium supplemented with 2,4-D (9µM), BAP (0.88µM) and IAA (2.85µM). Somatic embryos thus obtained, developed into plantlets within 30 days with 70% conversion rate. Zang et al. (2010) used mature zygotic embryos for inducing somatic embryogenesis and organogenesis in *D. hamiltonii*. Embryogenic calli were formed when explants were cultured in MS medium supplemented with 2,4-D (4.52-13.57 µM). NAA in combination with Kn and BAP effectively induced shoot differentiation from callus but after several subcultures the calli usually turned brown or even got necrotized. This problem could be resolved by depleting all hormones from culture medium after pre-differentiation (Yuan et al. 2009).

Somatic embryos offer faster rates of multiplication in bamboos and it is imperative to carry out molecular characterization of the somatic embryos at different morphogenetic stages to test clonal fidelity as has been done in *D. hamiltonii* (Sood et al. personal communication), *B. balcooa* (Gillis et al. 2007) and *B. nutans* (Mehta et al. 2010).

Table 2.1- Current status of tissue culture of bamboos

| Bamboo Species | Explant | Results | Reference |
|---|---|---|---|
| *Arundinaria gigantea* | Nodal segment | Multiple shoot formation | Baldwin et al. 2009 |
| *Bambusa arundinacea / Bambusa bambos* | Seeds | Somatic embryogenesis | Mehta et al. 1982 |
| | Nodal segment | Multiple shoot formation | Nadgir et al. 1984 |
| | Nodal segment | Multiple shoot formation and rooting | Arya et al. 1998 |
| | Seeds | Rhizome and plantlet formation | Kapoor and Rao, 2006 |
| *Bambusa balcooa* | Nodal explant | Multiple shoot formation and rooting | Das and Pal 2005a, b |
| | Nodal explant | Multiple shoot formation and rooting | Islam and Rahman, 2005 |
| | Mature pseudospiklets | Somatic embryogenesis | Gillis et al. 2007 |
| | Nodal explant | Multiple shoot formation and rooting | Mudoi et al. 2009 |
| *Bambusa beecheyana* | Inflorescence | Somatic embryogenesis | Yeh and Chang, 1986b |
| | Roots | Embryogenic calli | Chang and Lan, 1995 |
| *Bambusa edulis* | Nodal segment | Multiple shoot formation and rooting | Lin et al. 1998 |
| | Inflorescence | Somatic embryogenesis | Lin et al. 2004a |
| | Inflorescence | Inflorescence proliferation | Lin et al. 2005a |
| | Inflorescence | Plantlet formation and inflorescence proliferation | Lin et al. 2005b |
| *Bambusa oldhamii* | Young inflorescence | Somatic embryogenesis | Yeh et al. 1986a |
| | Shoot tip | Callus cultures | Huang et al. 1983 |
| | | Plantlets | Huang et al. 1989b |
| | | Callus | Huang et al. 1990 |
| | Nodal segment | Multiple shoot formation and rooting | Lin et al. 2007a |

| | | | |
|---|---|---|---|
| *Bambusa glaucescens* | Nodal segment | Multiple shoot formation and rooting | Banik, 1987 |
| | Nodal segment | Multiple shoot formation and rooting | Shirin and Rana, 2007 |
| *Bambusa multiplex* | Shoot tip | Callus | Huang et al. 1983 |
| | | Protoplast | Huang et al. 1989a |
| | | Plantlets | Huang et al. 1989b |
| | | Callus | Huang et al. 1990 |
| | Leaves | Embryogenic calli | Jullien and Van, 1994 |
| *Bambusa nana* | Nodal explant | Multiple shoot formation and rooting | Shirin and Arya, 2003 |
| *Bambusa nutans* | Seeds | Multiple shoot formation and rooting | Yashodha et al. 1997 |
| | Nodal explants | Multiple shoot formation and rooting | Islam and Rahman, 2005 |
| *Bambusa salarkhanii* | Nodal explants | Multiple shoot formation and rooting | Islam and Rahman, 2005 |
| *Bambusa vulgaris* | Nodal segment | Multiple shoot formation | Nadgir et al. 1984 |
| | Seeds | Somatic embryogenesis | Rout and Das, 1994 |
| | Nodal explant | Multiple shoot formation and rooting | Hirimburegama and Gamage, 1995 |
| | Shoots | Callogenesis and organogenesis | Rout and Das, 1997 |
| | Nodal explant | Somatic embryogenesis | Gielis, 1999 |
| | Nodal explant | Multiple shoot formation and rooting | Islam and Rahman, 2005 |
| | Nodal explant | Multiple shoot formation and rooting | Ramanayake et al. 2006; Ndiaye et al. 2006 |
| *Bambusa ventricosa* | Nodal explant | Multiple shoot formation | Dekkers and Rao, 1989 |
| | Culm sheath | Callus | Dekkers and Rao, 1989 |
| | Shoot apex | Plantlets | Huang and Huang, |

| | | | |
|---|---|---|---|
| | | | 1995 |
| | Nodal explant | Embryogenic calli | Gielis, 1999 |
| *Bambusa tulda* | Seedlings | Multiple shoot formation and rooting | Saxena, 1990 |
| | Nodal explants | Multiple shoot formation and rooting | Das and Pal, 2005b |
| | Nodal explants | Multiple shoot formation and rooting | Mishra et al. 2007 |
| *Bambusa wamin* | Nodal segment | Multiple shoot formation and rooting | Arshad et al. 2005 |
| *Dendrocalamus brandisii* | Nodal explants | Multiple shoot formation and rooting | Vongvijitra, 1988 |
| | Nodal explants | Flowering | Nadgauda et al. 1990 |
| | Nodal explants | Multiple shoot formation and rooting | Mukuntha kumar et al. 1999 |
| *Dendrocalamus asper* | Seeds | Plantlet formation | Arya et al. 1999 |
| | Nodal segments and Seeds | Multiple shoot formation and rooting | Arya et al. 2002 |
| | Inflorescence | Multiple shoot formation and rooting | Arya et al. 2008a |
| | Nodal explants and leaf bases | Somatic embryogenesis | Arya et al. 2008b |
| | Root, leaves and nodal segments | Somatic embryogenesis | Ojha et al. 2009 |
| *Dendrocalamus giganteus* | Nodal segment | Multiple shoot formation and rooting | Ramanayake et al.1997 |
| | Inflorescence | Multiple shoot formation | Ramanayake et al.1998 |
| | Shoot apex and young leaves | Embryogenic callus and plantlets | Fonseca et al. 1998 |
| | Mature seeds | Somatic embryogenesis | Rout and Das,1994 |
| | Nodal explant | *In vitro* flowering | Ramanayake et al. 2001 |
| | Shoots, spikelet and roots | organogenesis | Ramanayake et al. 2003 |
| *Dendrocalamus hamiltonii* | Nodal explant | Multiple shoot formation and rooting | Sood et al. 1992 |
| | Seedlings | Multiple shoot | Chambers et al. 1991 |

| | | | |
|---|---|---|---|
| | | formation, rooting and *in vitro* flowering | |
| | Nodal explant | Multiple shoot formation and rooting | Sood et al. 2002a |
| | Nodal explant | Somatic embryogenesis and floral bud induction | Godbole et al. 2002 |
| | Nodal explant | Multiple shoot formation and rooting | Agnihotri and Nandi, 2009; Agnihotri et al. 2009 |
| | Zygotic embryo | Somatic embryogenesis and organogenesis | Zhang et al. 2010 |
| *Dendrocalamus latiflorus* | Internode<br>Inflorescence | Plantlets<br>Inflorescence proliferation, multiple shoot formation and rooting | Zamora et al., 1988<br>Lin et al. 2006 |
| *Dendrocalamus longispathus* | Nodal explant | Multiple shoot formation and rooting | Saxena and Bhojwani, 1993 |
| *Dendrocalamus membranaceus* | Seeds | Multiple shoot formation and rooting | Yashodha et al. 1997 |
| *Dendrocalamus strictus* | Seedling | Multiple shoot formation and rooting | Kumar, 1994 |
| | Seedling | Multiple shoot formation and rooting | Nadgir et al. 1984 |
| | Nodal segment | Multiple shoot formation and rooting | Nadgir et al. 1984 |
| | Seeds | Somatic embryogenesis | Rao et al. 1985 |
| | Seedlings | Multiple shoot formation and rooting | Dekkers and Rao, 1989 |
| | Seedlings | Plantlets | Preetha et al. 1992 |
| | Nodal segment | Rooting only | Chaturvedi et al. 1993 |
| | Seeds | Somatic embryogenesis | Rout et al. 1994 |
| | Seedling | Multiple shoot formation and rooting | Shirgurkar et al. 1996 |

| | | | |
|---|---|---|---|
| | Seeds/seedlings<br><br>Nodal segment/ seedling<br>Seeds<br><br>Seedlings<br><br>Seeds | Multiple shoot formation<br><br>Multiple shoot formation and rooting<br>Somatic embryogenesis<br><br>Multiple shoot formation and rooting<br><br>Multiple shoot formation and rooting | Maity and Ghosh, 1997<br><br>Ravikumar, 1998<br><br>Saxena et al. 1999<br><br>Singh et al. 2001<br><br>Reddy, 2006 |
| *Guadua angustifolia* | Nodal explant | Multiple shoot formation and rooting | Jimenez et al. 2006 |
| *Gigantochloa atroviolaceae* | Nodal explant | Multiple shoot formation and rooting | Bisht et al. 2010 |
| *Octatea acuminata aztecorum* | Seeds | Somatic embryogenesis | Woods et al. 1992 |
| *Phyllostachys aurea* | Shoot tip | Callus | Huang et al. 1983 |
| *Phyllostachys meyeri* | Seedlings | Multiple shoot formation and rooting | Ogita et al. 2008 |
| *Phyllostachys nigra* | Shoots | Callus | Ogita et al. 2005 |
| *Pseudoxytenanthera stocksii* | Nodal explant | Multiple shoot formation and rooting | Sanjaya et al. 2005 |
| *Schizostachyum brachycladum* | Culm sheath | Callus | Dekkers and Rao, 1989 |
| *Sinocalamus latiflorus* | Seeds<br><br>Anthers | Somatic embryogenesis<br><br>Somatic embryogenesis | Yeh and Chang, 1987<br><br>Tsay et al. 1990 |
| *Thamnocalamus spathiflorus* | Nodal segment | Multiple shoot formation and rooting | Bag et al. 2000 |
| *Thyrsostachys oliveri* | Nodal explant | Multiple shoot formation and rooting | Islam and Rahman, 2005 |
| *Thyrsostachys Siamensis* | Culm sheath base | Callus | Dekkers and Rao, 1989 |

# Chapter 3
# Materials and Methods

# 3 Materials and methods

## 3.1 Micropropagation

Micropropagation of different species of bamboos namely *Dendrocalamus asper*, *Guadua angustifolia*, *Phyllostachys pubescens* and *Bambusa tulda* was included in the scope of present investigation.

**3.1.2 Explant source:** 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 4) Length of internode. Explants were collected from the field grown plants from the experimental garden of the Institute for the species *Dendrocalamus asper* and *Bambusa tulda.* For *Guadua angustifolia,* explants were taken from potted plants raised from seeds. Seeds of *Phyllostachys pubescens* were procured from China and germinated under *in vitro* conditions and explants were taken from *in vitro* raised seedlings.

**3.1.3 Chemicals/ Glassware/ Plasticware:** For micropropagation studies**,** all chemicals used were of Sd Fine/ Sigma/ Qualigens brands (AR grade). All glasswares were made up of borosilicate glass (Borosil glass Ltd, Mumbai; or Duran, Germany). Kasablanka jars were also used for routine sub -culturing work. 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, Banglore, India. PCR reactions were carried out in PCR tubes (Tarsons).

**3.1.4 Culture media:** For micropropagation, generally Murashige and Skoog's (1962) medium (MS), was used. Media stocks were prepared in double distilled water (Annexure - I). Sucrose 2% was used unless otherwise specified. The semi-

solid media were prepared with agar {0.75% (w/v), Qualigens, Mumbai} or Phytagel (0.25%, Sigma) and the pH was adjusted to 5.7 with either 0.1N HCl or 0.1N NaOH before autoclaving. The media were steam sterilized for 20 minutes at 1.1 kg/cm$^2$ and 121 $^0$C.

**3.1.5 Culture incubation**: Cultures were incubated at Photosynthetic Photon Flux density of 70 ± 5 mmol m$^{-2}$ s$^{-1}$ from cool white fluorescent lamps (Philips, India) at 25 ± 2 $^0$C. Day length maintained was of 16 h on a 24 h -light/dark cycle unless otherwise mentioned.

**3.1.6 Surface sterilization of nodal explants:** Nodal buds on secondary and tertiary branches were selected and used for initiating aseptic cultures. After removal of leaf sheaths, hair and surface cleansing of explants with liquid detergent (Rankleen, Ranbaxy, India) with a sable-hair brush, these were treated with an aqueous solution of 0.1% Bavistin and 0.04% streptomycin sulphate for about 20 minutes on a horizontal shaker (100 rpm) and rinsed with distilled water. Thereafter, the explants were treated with 70% alcohol for 2 minutes before repetitive washings in sterile distilled water. The explants were finally exposed to 0.1% mercuric chloride solution treatment with a drop of liquid detergent (Tween20-HIMEDIA) for 10- 15 minutes and again washed thoroughly with sterile distilled water in a laminar flow cabinet. On both the ends, a few mm portions of explants exposed to the sterilant were removed with the help of a sharp (sterile) secateur and the explants placed upright in test tubes containing MS medium supplemented with sucrose (2% or 3% w/v) and agar (0.8%, w/v) and various growth regulators.

All experimental manipulations were carried out under aseptic conditions in an inoculation chamber fitted with a bactericidal UV tube (15W, peak emission 2537Å). The floor of chamber was scrubbed thoroughly with cotton dipped in alcohol. The

surface of all the vessels and other accessories such as spatula, forceps, glass plate, needles and scalpel, tubes containing absolute alcohol etc. were also cleaned with spirit. Alcohol was sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with UV rays kept continuously on for one hour. Hands and arms which were to be introduced inside the chamber were scrubbed with alcohol before inoculation. The rims of test tubes/culture bottles and the sides of the plugs were flame sterilized. Instruments were also sterilized by dipping in rectified spirit and flaming a number of times. Care was taken to cool the instruments before putting into operation.

**3.2 Initiation of aseptic cultures**

Micropropagation work was carried out by exploiting forced axillary branching technique from nodal explants in all the four bamboos. Attempts were also made to raise plantlets through callus in *Dendrocalamus asper.* Various factors like initial response of explants, contamination rate, bud break and survival percentage were considered for initiation of cultures.

Explants tested for initiating cultures

1. Apical buds – *In vivo* growing apices of primary and secondary shoots were used to raise cultures
2. Nodal buds scooped out from nodes – remained alive for a long time but were unable to induce growth
3. Nodal explants - were found to be most suitable for raising multiple shoots as well as for raising callus cultures.
4. Leaf explants - Leaf explants from both *in vivo* and *in vitro* raised plants were used but no growth could be induced in them.

**3.2.1 Effect of season and source of explant**: Nodal explants were collected from January 2008 to Dec 2008 on a regular basis to study the effect of season on the desired response of the explants. 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 BAP (as per requirement), 2% or 3% sucrose (as per requirement) and 0.8% agar for initial screening. Percent survival was tested on tenth day basically for asepsis and bud break.

**3.3 Shoot proliferation**

The sprouts were excised from the explants and transferred to both agar-gelled and static liquid media supplemented with different concentrations and combinations of cytokinins. For shoot proliferation, different concentrations and combinations of cytokinins were used for either of the bamboo species depending upon their response and requirement. Regular sub-culturing was resorted to every three weeks. Dead decaying tissue was removed during each sub-culturing. The data in terms of number of shoots formed was recorded after 30, 45, 60 and 90 days respectively. Data in terms of length of longest shoot, number of shoots formed was also recorded. Details of media combinations used for axillary shoot proliferation are described below-

1) MS (0)

2) MS + BAP (2.26-22.15 µM)

3) MS + BAP (4.43-13.29 µM)+ AdS (2.7-16.20 µM)

4) MS + BAP (4.43 µM-13.29 µM) + Kn (2.32 µM-9.3 µM)

5) MS + Kn (2.32 µM-9.3 µM)

### 3.3.1 Propagules for multiplication

After first cycle of multiplication, the shoots were divided into clumps of 3,6,9 and 12 shoots and the effect of number of shoots per propagule was evaluated by inoculating shoot clumps onto the multiplication medium.

### 3.3.2 Carbon source

To study the effect of carbon source on shoot multiplication, the clumps of shoots were also inoculated onto multiplication medium supplemented with 2% sucrose, glucose and table sugar respectively. Best carbon source was selected on the basis of number of shoots formed and length of shoots achieved**.**

### 3.3.3 Gelling agent

Effect of various gelling agents like phytagel, agar and liquid medium on multiple shoot proliferation was also studied.

**3.4 Rooting of microshoots**: To achieve rooting of microshoots, various auxins were tried either singly or in different combinations. Cut ends of the microshoots in bunches of 2-3 were placed in MS medium supplemented with auxins. The details of PGR combinations used for root induction are given below:

1) MS + IBA (2.46-24.60 µM)

2) MS + NAA (3.67-14.7 µM)

3) MS + IBA (2.46-24.60 µM)+ NAA (3.67-14.70 µM)

4) MS + IAA (2.85-28.55 µM)

5) MS + IBA (2.46-14.76 µM) + IAA (2.85-17.13 µM)

6) MS + IBA (2.46-24.60 µM) + Coumarin (6.84-68.4 µM)

### 3.5 Acclimatization

Rooted plantlets were washed thoroughly in lukewarm water with the help of a soft, sable-hair brush for removal of agar adhering to the plantlets growing on the gelled

medium. These were transferred to plastic pots (4" dia.) containing wet sand and kept in a poly/greenhouse, covered initially with inverted glass beakers for 8–10 days for acclimatization. These were then transferred to the potting mixture.

Proper potting mixture was selected by transferring the plants in various potting mixes as given below:

$PM_1$ - sand:soil:manure :: 1:1:1

$PM_2$ - sand:soil:manure :: 1:1:2

$PM_3$ - sand:soil:manure :: 1:0:0

$PM_4$ - sand:soil:manure :: 0:0:1

**3.6 Callus induction**

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 2% sucrose unless otherwise mentioned and the details of combinations employed for callus induction are as follows:

1) MS + 2,4-D (2.43-38.96 µM)

2) MS + NAA (3.67-14.7 µM)

3) MS + BAP (2.26-8.86 µM)

4) MS + 2,4-D (2.43-24.35 µM) + NAA (3.67-14.7 µM)

5) MS + 2,4-D (2.43-24.35 µM) + BAP (2.26-8.86 µM)

6) MS + 2,4-D (2.43-38.96 µM) + CM (10%)

**3.6.1 Effect of MS strength on callus induction**

To test the effect of strength of MS major salts on callus induction, the following four strengths of MS were tried:

MS full strength, ¾ MS, ½ MS and ¼ MS

### 3.6.2 Callus proliferation

To find the best medium for callus proliferation, creamish white nodular callus was shifted to MS medium supplemented with various combinations of auxins and cytokinins. Growth rate of callus was calculated by inoculating 2g of callus on the different multiplication media and fresh weight of callus was calculated after 30 days and best medium was selected based on maximum growth of the callus.

### 3.6.3 Regeneration of shoots and roots 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.

### 3.6.4 Statistical analysis

The effect of different treatments was determined by analysis of variance (ANOVA) using STATISTICA data analysis software v7 (StatSoft Inc., Tulsa, OK). Significance differences between the means were assessed by Duncan's Multiple Range Test (DMRT) at p=0.05.

### 3.6.5 Microtomy:

i) **Fixing and Killing of material**: For ascertaining different morphogenetic stages in *Dendrocalamus asper,* callus lumps were fixed in a freshly prepared fixative FAA (Formalin: glacial acetic acid: 50% ethanol :: 5:5:90 ) and subsequently in 70% ethanol until use after which the tissue was dehydrated in the following TBA (t-butyl alcohol) series:

Ethanol(ml) : TBA (ml) : Water

a) 30 Ethanol: 20 TBA : 50 Water

b) 50 Ethanol: 20 TBA: 30 Water

c) 50 Ethanol: 35 TBA : 15 Water

d) 45 Ethanol : 55 TBA

e) 25 Ethanol : 75 TBA

f) 100 TBA

The material was kept in each grade for 3-4 h except for 'c' where it was kept overnight.

ii) **Waxing**: For waxing, the tissue in TBA was kept in an oven preset at $60^0$C and paraffin wax flakes were added after every 15-20 minutes. 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. Dewaxing was done in the following grades and sections stained in safranin and fast green dyes and permanent slides were made using a transparent moutant DPX.

a) 75 ml Xylol: 25 ml Ethanol

b) 50 ml Xylol: 50 ml Ethanol

c) 25 ml Xylol: 75 ml Ethanol

d) Ethanol

e) 25 ml water : 75 ml ethanol

f) 50 ml water : 50 ml ethanol

g) 75 ml water : 25 ml ethanol

h) Safranin (6-24 h)

i) 75 ml water : 25 ml ethanol

j) 50 ml water : 50 ml ethanol

k) 25 ml water : 75 ml ethanol

l) Ethanol-1

m) Ethanol-2

n) Clove oil 25% in ethanol

o) Clove oil 50% in ethanol

p) Fast green (prepared in 50% clove oil)

q) Clove oil 50% in Xylol

r) Clove oil 25% in Xylol

s) Xylol-1 (30 minutes)

t) Xylol-2 (30 minutes)

u) Mounted the slides in D.P.X. mountant

Sections were examined under the light microscope (Nikon-Labphot-2) and photographed by means of an automatic photomicrography system.

**3.7 Clonal fidelity of *in vitro* raised plants**

**3.7.1 Protocol for DNA isolation**: DNA isolation was carried out following the base protocol of Saghai-Maroof *et al*. (1984) with slight modification as described by Singh *et al*. (1999).

1 2-3 g of leaves taken from *in vitro* raised field established plants were grounded to a fine powder in a pestle and mortar in the presence of liquid nitrogen.

2 Transferred the powder to a microfuge tube containing 15 ml of CTAB extraction buffer (which was maintained at 65 $^{0}$C in a water bath) and mixed vigorously.

3 It was then incubated at 65 $^{0}$C for 1 h with occasional mixing.

4 Cooled the mixture to room temperature and then added equal volume of chloroform : isoamyl alcohol (24:1) and mixed gently by inverting the tubes for 2 minutes.

5 Centrifuged at 10,000 rpm for 10 minutes at room temperature (25 $^{0}$C).

6 Transferred the clear supernatant to a fresh tube. Repeated the chloroform : isoamyl alcohol extraction.

7 Precipitated the DNA by adding 0.6 volume (600 ml) of isopropanol. Mixed gently by inversion and left at room temperature for about 10 minutes.

8 Centrifuged the precipitated DNA at 13,000 rpm for 15 minutes at room temperature.

9 Decanted off the supernatant, added l ml of 70% ethanol and repeated the above step.

10 Dried the pellet at room temperature or 37 $^{0}$C for about 1 h and dissolved the DNA in 1 ml TE buffer.

11 Treated the DNA with 3 µl of RNase A (10 mg/ml stock) for 45 minutes at 37 $^{0}$C.

12 Extracted once with an equal volumes of phenol: chloroform: isoamyl alcohol.

13 Transferred the aqueous phase to a new tube and extracted once again with chloroform : isoamyl alcohol.

14 Transferred the upper aqueous phase to a fresh tube and precipitated the DNA by adding 1/10$^{th}$ volume of 3 M Sodium acetate (pH 4.8) and 2 volumes of chilled absolute ethanol. Left at -20 $^{0}$C for half an hour to overnight.

15 Pelleted the DNA by centrifugation at 13,000 rpm for 15 minutes at 4 $^{0}$C. Washed the pellet once with 70% ethanol to remove the extra salts by centrifuging at room temperature.

16 Dried the pellet at room temperature or in a vacuum desiccator and dissolved the DNA in minimum volume of TE (10:1) buffer (1 ml) and stored at -20 $^{0}$C until used.

**3.7.2 Quantification of the 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 A260/A280 = 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

**3.7.3 Optimization of PCR parameters:**

For RAPD, three different annealing temperatures viz. 35 $^0$C, 36 $^0$C and 37 $^0$C were tested for better amplification.

The reaction buffer for ISSR consisted of 2.5 µl Taq buffer, 1 µl $MgCl_2$, 0.15 µl dNTPs (10mM each of dATP, dGTP, dTTP and dCTP), 1.5 µl primer, 0.17 µl Taq polymerase and 17.68 µl water. PCR amplification was performed in a Bio Rad I cycler from Bio Rad Laboratories (India) Pvt. Ltd. which was programmed for initial DNA denaturation at 94$^0$C for 4 minute, followed by 44 cycles of 1 minute denaturation at 94$^0$C, 1 minute annealing (temperature specific to the primer) and 1 minute extension at 72$^0$C, with a final extension at 72$^0$C for 7 minutes.

For RAPD, reaction buffer consisted of 2.5 µl Taq buffer, 0.5 µl $MgCl_2$, 0.2 µl dNTPs, 1.5 µl primer, 0.17 µl Taq polymerase and 18.13 µl water. PCR amplification consisted of initial denaturation at 94$^0$C for 5 minutes, followed by 45 cycles of 1 minute denaturation at 94$^0$C, 1 minute annealing at 37$^0$C and 2 minutes extension at 72$^0$C, with a final extension at 72$^0$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

images were taken with the help of Bio Rad Molecular Imager (Gel Doc TM XR imaging system from Bio Rad Laboratories, India, Pvt. Ltd).

### 3.7.4 Scoring of bands

For each primer, bands were scored for the presence or absence 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).

### 3.8 Isolation and identification of bacteria from *Guadua angustifolia* cultures

Bacterial contamination was invariably encountered in all the cultures of Guadua angustifolia after 20 days of planting. The contaminating bacteria were picked up with a loop from visibly contaminated cultures and were inoculated directly on the LB medium (Himedia). After incubation at 25$^{o}$C for 24 hours, two types of colonies were observed. Pure cultures of these bacteria were obtained by picking up the colonies and streaking them onto the fresh medium. These cultures were further maintained in glycerol stock at –80$^{o}$C.

Bacterial DNA was isolated from pure culture with Genelute Bacterial Genomic DNA Isolation Kit (Sigma) and PCR amplifications were performed. The sequence of primers used for amplification were 5′-AGAGTTTGATCATGGCTCAGA- 3′ and 5′ GTTACCTTGTTACGACTT-3′-corresponding to 8 to 28 and 1493 to 1510 respectively, which are parts of 16S rRNA gene of *Escherichia coli* and are useful for amplifying 16S rRNA gene from various kinds of bacteria (Salvan et al.2010). Each 50 µl PCR reaction mixture contained: 1 µl (10 µM) of each primer, 2 µl (50–100 ng) template DNA, 0.5 µl (10 mM) dNTPs, 5 µl (10X) reaction buffer and 0.5 µl (5.0 U/µl) of Taq polymerase (Sigma). All reaction mixtures were incubated in a thermal cycler (Perkin Elmer Gene Amp System 2400) for 4 minutes at 95 °C and then subjected to

35 amplification cycles of 1.0 minute at 95 °C, 1.0 minute at 52 °C, and 2 minutes at 72 °C followed by 8 minutes final extension at 72 °C. The amplicons were separated on 1.0% (w/v) agarose gel stained with ethidium bromide.

The 16S rRNA gene of bacteria was further sequenced to analyze its identity. Briefly, the amplified 16S rRNA gene was purified from the agarose gel using a Nucleospin Extract II kit. The PCR-purified product was directly used for nucleotide sequencing of the gene by using a Big Dye$^{R}$ Terminator Cycle sequencing kit (Applied Biosystems). To identify bacteria, preliminary searches in the NCBI database were performed with BLASTIN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA).

### 3.8.1 Selection of suitable antibiotic

The luria agar plates containing different antibiotics like kanamycin (Sigma), carbenicillin (HiMedia), ampicillin (Sigma), rifampicin (HiMedia), and streptomycin sulfate (Sigma) were inoculated with isolated bacterial contaminants for antibiotic screening and the best antibiotic was selected on the basis of its effectiveness in antibiotic sensitivity testing.

### 3.8.2 Treatment of Contaminated shoots

The antibiotics were added to the multiplication medium in the following dosages: 0, 5, 10, 15, 25, 40, 50 μg/ml either alone and in combinations. Contaminated explants were then dipped in this medium for 10 days and best concentration of antibiotic was selected.

# Chapter 4
# Results

# 4 Results

## Objective 1- To develop and standardize mass propagation of *Bambusa tulda*, *Dendrocalamus asper*, *Guadua angustifolia*, *Phyllostachys pubescens* under *in vitro* conditions.

## 4.1 Micropropagation studies in *Dendrocalamus asper* Backer Ex K. Heyne

### 4.1.1 Initiation of aseptic cultures

#### 4.1.1.1 Selection of elite mother plant

Prior selection of the mother plant is a very important step while carrying out micropropagation studies. In the present study, selection of mother plant was done by taking into the consideration the following important parameters:1) Height of bamboo culm 2) Girth of culm at third internode from the bottom 3) Number of culms per clump 4) Length of internode

Explants were taken from the precocious branches of five year old field grown plant.

#### 4.1.1.2 Choice of explants

Various explants namely apical buds, leaves and young buds excised from nodes and nodal segments were initially tested for initiating cultures.

Explants were subjected to repeated washings under running tap water for 30 minutes to remove all the adhering dust particles and microbes from the surface. The explants were then cleaned with liquid detergent (Tween20-HIMEDIA) with the help of a sable hair brush and then washed properly with distilled water to remove the traces of detergent. After that the explants were treated with a suitable fungicide like bavistin and an antibiotic (Streptomycin sulphate) for another 20 minutes to remove fungus and bacteria respectively. Different concentrations of bavistin (0.1-1%) and

streptomycin sulphate (0.02-0.1%) were tried for surface sterilization but 0.1% bavistin and 0.04% streptomycin sulphate were found to be the most effective.

Under sterile conditions in a laminar air flow cabinet, the explants were treated with 70% alcohol for one minute followed by a treatment with $HgCl_2$ solution (0.04% w/v) for 6 minutes. The explants were then inoculated onto MS basal medium containing 2% sucrose for culture initiation.

Apical buds of secondary and tertiary branches from field grown selected elites were used for initiating cultures but they could not sustain growth and turned necrotic as they were unable to bear surface sterilization treatment. Similarly, young buds excised from secondary and tertiary branches planted on culture medium remained alive for one month but failed to sustain growth thereafter. Likewise, leaf explants did not show response on any of the media tried. However, the nodal explants taken from secondary and tertiary branches proved to be the most favorable for initiating aseptic cultures as they responded favorably to different media combinations.

#### 4.1.1.3 Seasonal effect on culture initiation

Due to the considerable variations in the environmental conditions during different periods of the year, maturity status of the explants varied with season, hence, the response of explants to the culture initiation also varied. During active growth from June to August, the explants were found to be tender and unable to bear the harsh sterilization treatments. On the other hand, during the periods of slow growth, appropriately hardened explants were available which could endure the sterilization treatment. Further, breaking the dormancy of buds varied with their position on the plant and season of the year as midculm nodes of tertiary branches were found to be the best explants for axillary shoot proliferation. In this case, best period for initiating

aseptic cultures were January and February when maximum bud break was achieved.

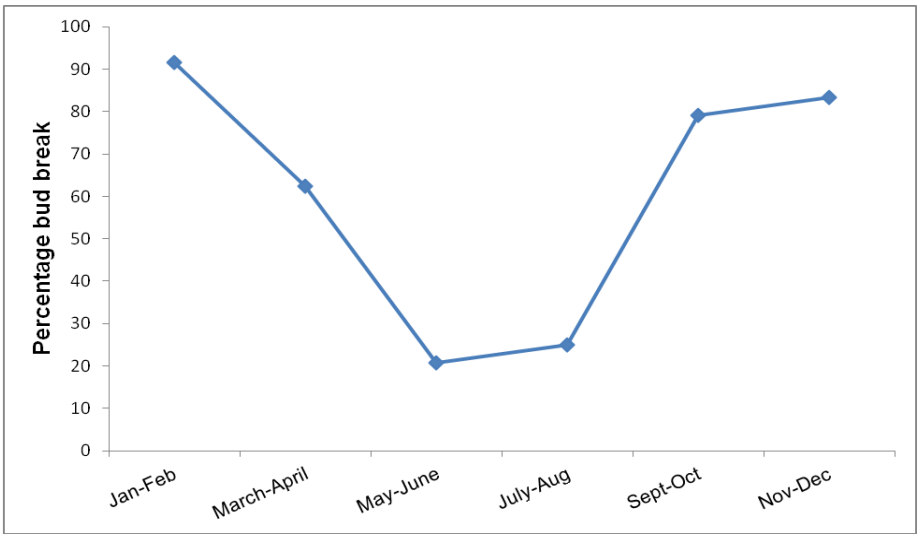



Figure 4.1.1 Variation in bud-break in nodal segments from January to December (2008)

**4.1.1.4 Contamination**

The rate of contamination in the cultures also varied with season. Rainfall had a direct influence on contamination rates and survival percentage of explants. March-April and November-December were observed to be the best months for initiating aseptic cultures because of low rates of contamination. In contrast, the highest rate of contamination was observed during the period of maximum rainfall (June-August). The prior screening of explants by inoculating them on half strength MS medium supplemented with 3% sucrose for 7-10 days proved to be beneficial.

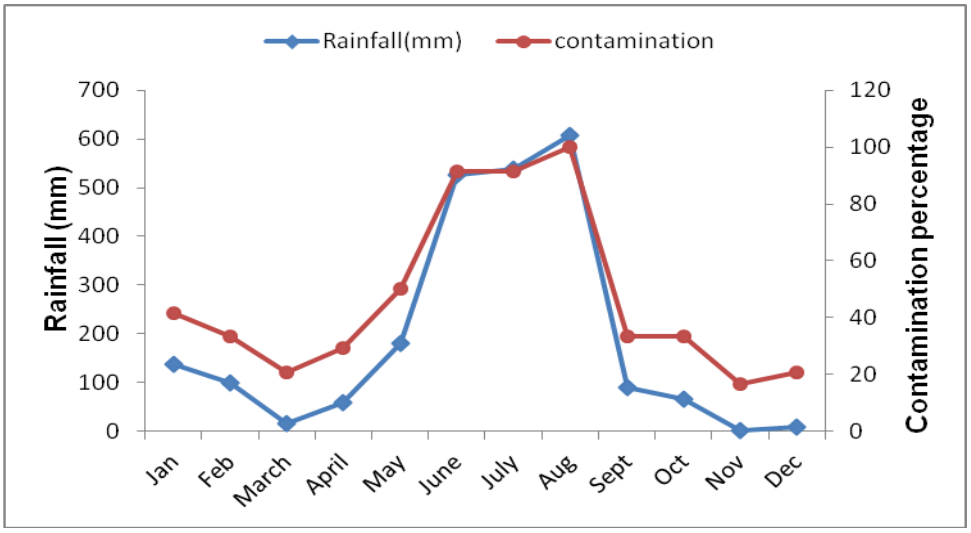



Figure 4.1.2 Variation in contamination percentage with rainfall from January to December (2008)

### 4.1.2 Establishment of aseptic cultures

For inducing sprouting, nodal explants were inoculated on MS medium with or without any cytokinins. Nodal explants cultured on MS basal medium without any cytokinin took more time to sprout (25 days) and that too with low efficiency (30%). However, the nodal explants sprouted within 15 days of inoculation on MS medium supplemented with BAP (8.86 µM) in 90% of the cultures (Figure 4.1.3). The sprouting response declined with increase (13.29 µM) as well as decrease (4.43 to 2.26 µM) in the concentration of BAP. Nodal explants failed to respond on kinetin supplemented MS medium.

The sprouted buds were excised from the nodal segments and transferred onto MS medium containing different concentrations and combinations of cytokinins like BAP(2.26-22.15 µM), Kn (2.32-13.95 µM) and adenine sulfate (1.35-27 µM) for inducing axillary shoot proliferation. The number of sprouted buds at each node was treated as individual shoot during present investigation. Preliminary experiments on MS medium supplemented with 3% sucrose led to necrosis and death of shoots. This necessitated the inclusion of cytokinins in the shoot multiplication medium either alone or in combinations. Among the various cytokinins employed for shoot

proliferation, BAP (8.86 µM) in conjunction with adenine sulfate (13.5 µM) was found to be the best where an average of 48.66 shoots were formed after 60 days (Figure 4.1.8). Higher concentrations of BAP or adenine sulfate not only lowered the multiplication rate but also resulted in the formation of stunted shoots. Addition of kinetin alone did not result in any shoot proliferation. The shoots remained dormant for some time and ultimately died. The details of the effect of different cytokinins on shoot proliferation are depicted in Table-4.1.1

Table-4.1.1 Effect of different cytokinins on shoot proliferation

| S.No | Treatment | No. of shoots after 45 days | No. of shoots after 60 days |
|---|---|---|---|
| 1 | BAP(4.43 µM) | 6.66 ± 0.88 $^{no}$ | 9.33 ± 0.66 $^{m}$ |
| 2 | BAP(8.86 µM) | 10.66 ± 0.66 $^{lm}$ | 13.66 ± 0.33 $^{jk}$ |
| 3 | BAP(13.29 µM) | 12.33 ± 0.88 $^{kl}$ | 14.66 ± 0.66 $^{j}$ |
| 4 | BAP(22.15 µM) | 5.00 ± 0.57 $^{op}$ | 6.33 ± 0.33 $^{op}$ |
| 5 | BAP(8.86 µM)+AS(2.7 µM) | 20.33 ± 0.33 $^{i}$ | 24.66 ± 0.33 $^{h}$ |
| 6 | BAP(8.86 µM)+AS(6.75 µM) | 25.66 ± 0.66 $^{h}$ | 33.66 ± 0.33 $^{f}$ |
| 7 | BAP(8.86 µM)+AS(10.8 µM) | 29.33 ± 0.88 $^{g}$ | 39.33± 0.88 $^{cd}$ |
| 8 | BAP(8.86 µM)+AS(13.5 µM) | 37.66 ± 1.45 $^{de}$ | 48.66 ± 1.33 $^{a}$ |
| 9 | BAP(8.86 µM)+AS(16.20 µM) | 37.00 ± 1.52 $^{e}$ | 45.66 ± 0.88 $^{b}$ |
| 10 | BAP(13.29 µM)+AS(16.20 µM) | 33.66 ± 0.88 $^{f}$ | 41.00 ± 0.57 $^{c}$ |
| 11 | BAP(8.86 µM)+Kn(2.32 µM) | 7.00 ± 0.57 $^{no}$ | 8.66 ± 0.66 $^{mn}$ |
| 12 | BAP(8.86 µM)+K(4.65 µM ) | 4.33 ± 0.33 $^{p}$ | 5.66 ± 0.33 $^{op}$ |
| 13 | BAP(8.86 µM)+Kn(9.3 µM) | 0.00 ± 0.00 $^{q}$ | 0.00 ± 0.00 $^{q}$ |
| 14 | Kn(2.32 µM) | 0.00± 0.00 $^{q}$ | 0.00 ± 0.00 $^{q}$ |
| 15 | Kn(4.65 µM ) | 0.00 ± 0.00 $^{q}$ | 0.00 ± 0.00 $^{q}$ |

Duncan test; p=0.05; Values followed by the same letters in superscript within the column are not significantly different.

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 (1.23-9.84 µM), IAA (1.42-11.42 µM) and NAA (1.83-14.7 µM). It was observed that the addition of any auxin into the multiplication medium considerably reduced the proliferation rate, although they increased the length of shoots. Hence, their addition to the multiplication medium was discontinued. After the optimization of various combinations and concentrations of cytokinins for shoot proliferation, the effect of various gelling agent such as agar (0.8%) and phytagel (0.25%) was also studied. In the present study, replacement of the solidified medium with liquid medium during multiple shoot proliferation did not prove effective. Use of phytagel instead of agar was found to be more beneficial for promoting shoot multiplication.

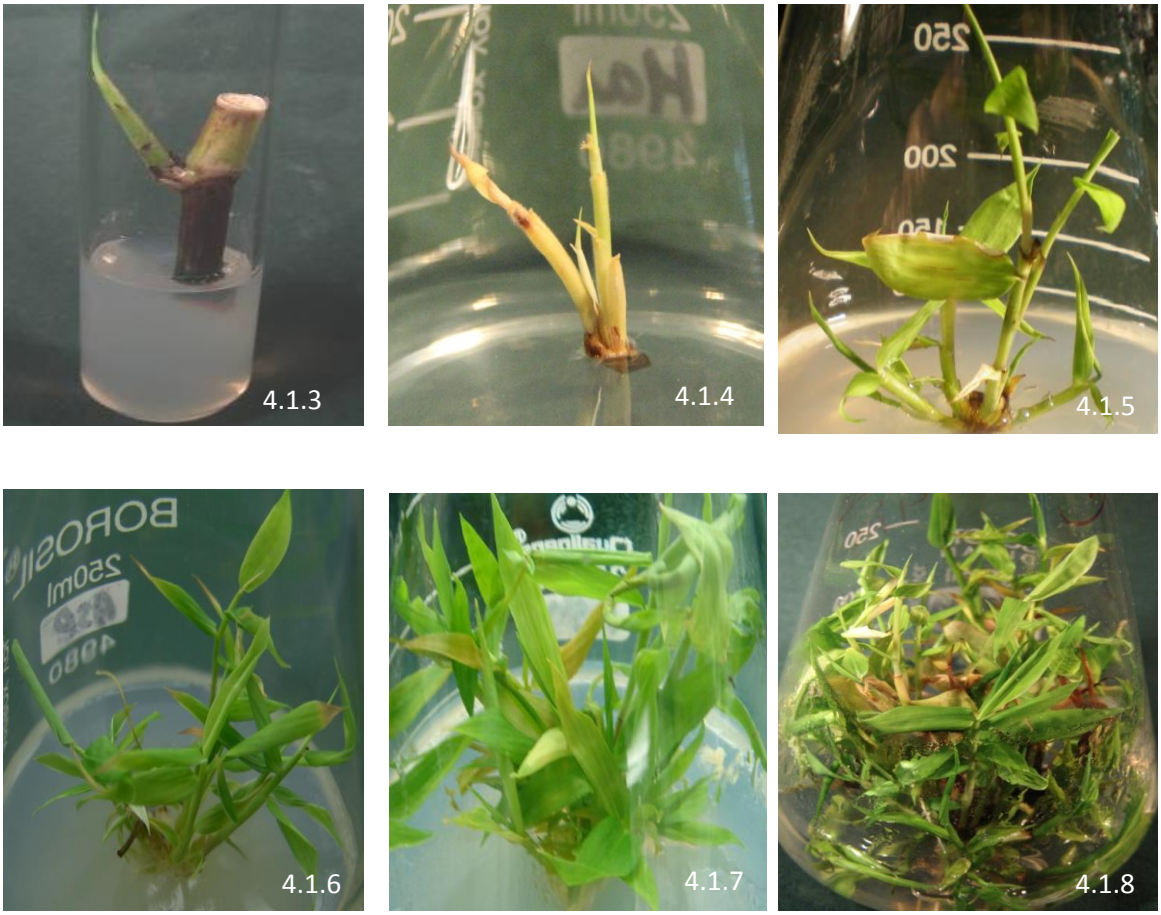



Figure 4.1.3-Axillary bud break on BAP(8.86 µM) after 15 days of inoculation; Figures 4.1.4 and 4.1.5- Initiation of multiple shoots on BAP (8.86 µM) and adenine sulphate (13.5 µM) after 20 and 30 days respectively; Figures 4.1.6 and 4.1.7- Multiple shoot proliferation after 45 days; Figure 4.1.8- after 60 days.

#### 4.1.2.1 Propagules for carrying out multiplication

Propagule used for recurrent multiplication of shoots is a critical factor during *in vitro* studies. In the present study, shoot clumps rather than single shoots were observed to be effective for multiplication of bamboo plants. The *in vitro* raised shoots were divided into clumps of three, six, nine and twelve shoots each and the effect of number of shoots (3,6,9,12) per propagule inoculated on multiplication medium was evaluated by culturing the clumps on MS medium supplemented with BAP(8.86 µM)and adenine sulfate(13.5 µM). Six shoots per propagule were found most effective for further multiplication where 27.2 shoots were obtained after 4 weeks of culture (Figure 4.1.9). However, use of more number of shoots per propagule (9,12) reduced multiplication rate as well as length of shoots formed.

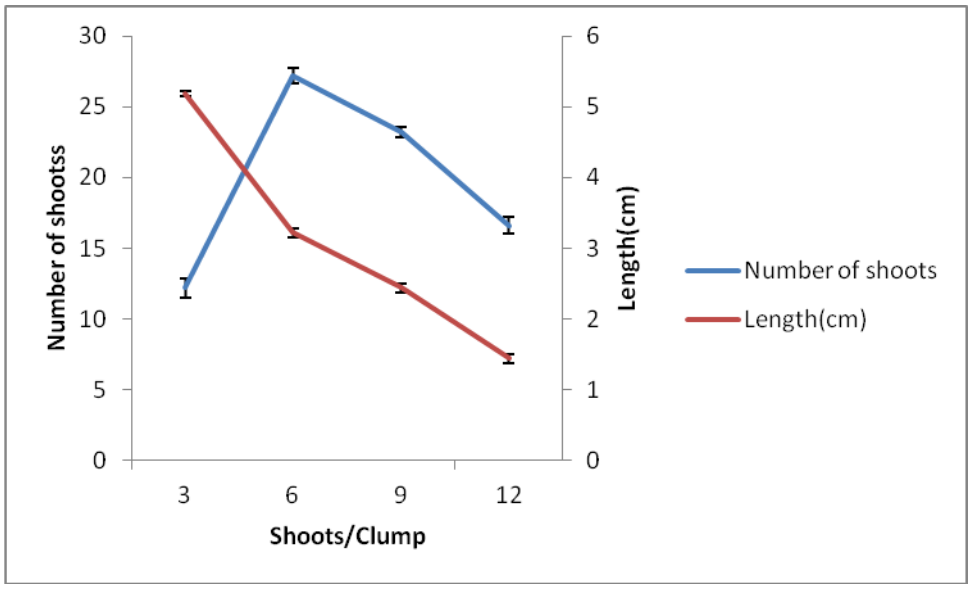



Figure 4.1.9- Effect of the number of shoot/clump on shoot multiplication in *D. asper*

#### 4.1.2.1 Effect of carbon source on multiplication rate

To study the effect of different carbon sources on shoot multiplication, six shoots per propagule were cultured on MS medium containing BAP (8.86 µM) and adenine sulfate (13.5 µM) supplemented with either 3% sucrose, glucose or table sugar respectively. Sucrose was found to be most suitable carbon source, as on an average 48.2 shoots were obtained after 6 weeks of culture (Figure 4.1.10). Length of shoots was also maximum (3.22 cm) in sucrose supplemented medium. However, 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.

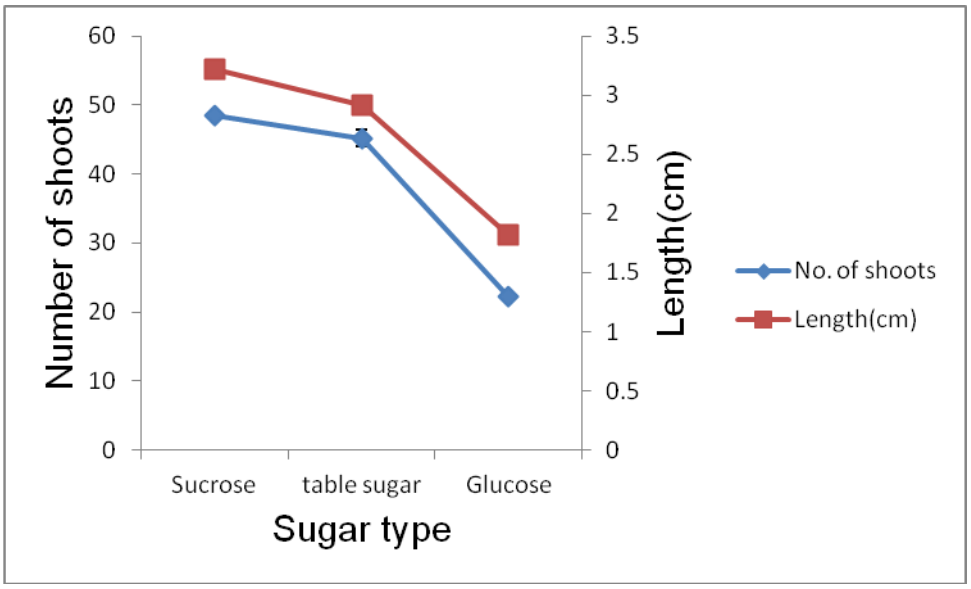



Figure 4.1.10- Effect of Carbon source on shoot multiplication

### 4.1.3 Rooting

Rooting is a major bottleneck while carrying out *in vitro* multiplication of bamboos. 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 onto MS medium supplemented with different concentrations and combinations of auxins. Shoots failed to root even after 45 days of culture on MS medium when

supplemented with auxins like IAA and NAA. Although, rooting was observed on IBA supplemented medium but response to rooting was delayed. On lower concentration of IBA (2.46 µM) only 10% rooting was observed. Rooting percentage improved with increase in concentration of IBA with maximum rooting (50%) on 14.76 µM IBA supplemented medium. With further increase in the concentration of IBA, development of roots declined and propagules did not survive. Addition of NAA (3.67 µM) to IBA (14.76 µM) supplemented medium proved to be very effective as maximum rooting response of 90% was observed with an average of 5.66 roots per propagule and mean root length of 3.06 cm after 45 days of culture. After 15 days of transfer to root induction medium, the propagules turned brown in all treatments. Hence, a regular transfer to the fresh media was done after every 15 days to ensure development of healthy shoots and roots.

Table 4.1.2- Effect of different auxins on root induction and length of roots

| S.no | Treatments | No. of roots formed | Length of roots(cm) |
|---|---|---|---|
| 1 | IBA (2.46 µM) | 0.33 ±0.33 $^{e}$ | 0.06±0.66 $^{g}$ |
| 2 | IBA (4.92 µM) | 1.66±0.33 $^{de}$ | 0.45±0.02 $^{f}$ |
| 3 | IBA (9.84 µM) | 2.66±0.33 $^{cd}$ | 0.47±0.01 $^{f}$ |
| 4 | IBA ( 14.76 µM) | 3.00±0.00 $^{cd}$ | 0.54±0.04 $^{f}$ |
| 5 | IBA (4.92 µM)+ NAA ( 3.67 µM) | 3.00±0.00 $^{bc}$ | 1.23±0.01 $^{e}$ |
| 6 | IBA (9.84 µM)+ NAA (3.67 µM) | 4.00±0.57 $^{a}$ | 2.17±0.05 $^{c}$ |
| 7 | IBA (14.76 µM)+ NAA (3.67 µM) | 5.66±0.33 $^{d}$ | 3.06±0.02 $^{a}$ |
| 8 | IBA (24.60 µM)+ NAA ( 3.67 µM) | 5.33±0.33 $^{ab}$ | 1.65±0.02 $^{d}$ |
| 9 | IBA (14.76 µM)+ NAA (7.35 µM) | 5.33±0.33 $^{ab}$ | 2.77±0.01 $^{b}$ |

Duncan test, p=0.05; Values followed by the same letters in superscript within the column are not significantly different



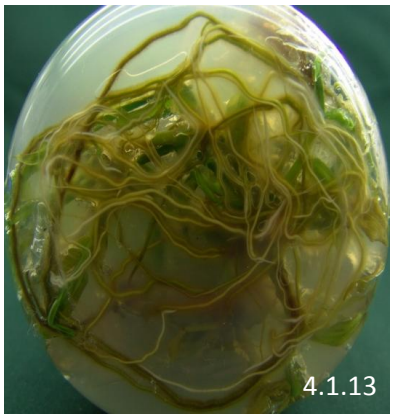




Figure 4.1.11– Root formation on the combination of IBA (14.76 µM) and NAA (3.67 µM) after 30 days; Figure 4.1.12- After 60 days; Figure 4.1.13- After 90 days of culture respectively; Figure 4.1.14- Complete plantlets formation before acclimatization.

### 4.1.4 Acclimatization

Plantlets were taken out of flasks and washed with luke warm water to remove agar sticking to them. These were then transferred to plastic pots containing river bed sand and covered with plastic jars for maintaining high relative humidity (80-85%). After 25 days, the plantlets were transferred into potting mixture containing sand:soil:farmyard manure in different ratios (v/v;1:1:1; 1:1:2; 1:0:0; 0:0:1 ) and kept in green house. Under green house conditions, foliage was sprayed with Hoagland solution after every 7 days. After one month of transplantation, the percentage survival was 95% in the 1:1:1 sand:soil:manure mixure, 90% in 1:1:2

sand:soil:manure mixture, 80% in sand and 60% in manure respectively . Hardened plants were finally transplanted in the field to pits (2ft×2ft×2ft) at a plant to plant and row to row distance of 6 meters. They were successfully established under field conditions and 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 4.1.16 a,b,c).



Figure 4.1.15-Acclimatized plants of *D. asper* in the green house; Figure 4.1.16 a,b,c-Hardened plants under field conditions after 3 months of transfer.

### 4.1.5 Callus induction

*In vitro* raised shoots were excised into small pieces (0.5-1cm) 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. Callus formation started at the cut ends after 15 days (Figure 4.1.17) and within 8 weeks, the whole explants turned into mass of callus (Figure 4.1.18). Addition of NAA or BAP proved ineffective for callus induction. MS medium supplemented with 2,4-D (14.61 µM) was found to be the best for inducing nodular and compact callus which was capable of sustained growth on subculturing on the same medium (Figure 4.1.19). At lower concentration of 2,4-D (2.43 µM), callus initiation was slow and took around 30 days to form callus. 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. 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. Effects of various PGRs on callus induction are given in the Table-4.1.3.

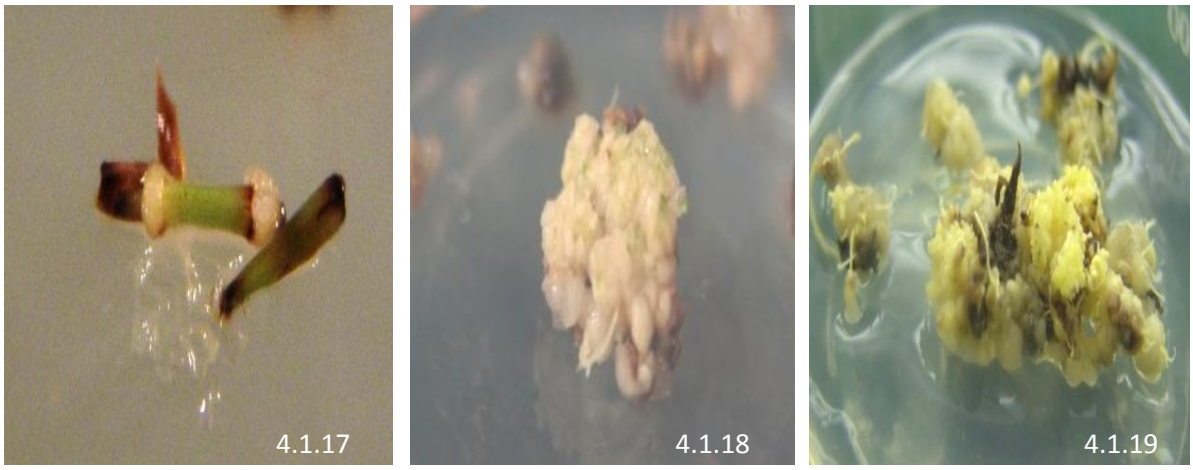



Figure 4.1.17- Induction of callus from nodal segment of *in vitro* raised shoots on 2,4-D (14.61 µM); Figure 4.1.18 and 4.1.19-Proliferation of callus after 60 days and 90 days respectively.

Table 4.1.3- Effect of various media combinations on callus induction from nodal explants

| S.No. | Media composition | Percent callus induction | Days required |
|---|---|---|---|
| 1 | MS | 0 | - |
| 2 | MS+NAA(14.7 µM) | 0 | - |
| 3 | MS+BAP(8.86 µM) | 0 | - |
| 4 | MS+2,4-D(4.87 µM) | 23.33 | 30 |
| 5 | MS+2,4-D(9.74 µM) | 53.33* | 20 |
| 6 | MS+2,4-D(14.61 µM) | 76.66* | 15 |
| 7 | MS+2,4-D(24.35 µM) | - | - |
| 8 | MS+2,4-D(14.61 µM)+BAP(2.26 µM) | 26.66 | 22 |
| 9 | MS+2,4-D(14.61 µM)+BAP(4.43 µM) | 20 | 24 |
| 10 | MS+2,4D(14.61 µM)+NAA(3.67 µM) | 40$^{\$}$ | 22 |

(Paired t-test; *,$^{\$}$ P < 0.05)

### 4.1.5.1 Effect of strength of MS medium on callus induction

Strength of basal MS medium was found to have an effect on callus induction percentage even when the concentration of 2,4-D remained same. It was observed that full strength MS medium gave better response with capability to induce callus in 76.66±3.33 % of cultures within 15 days of inoculation. Response to callus induction was least on ¼ MS medium (13.33±3.33%).

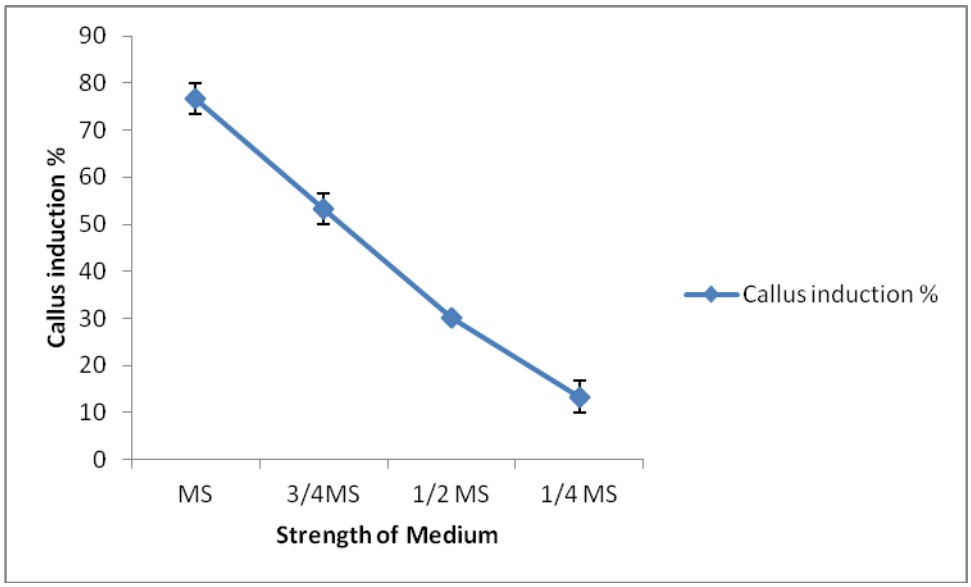



Figure 4.1.20- Effect of medium strength on callus induction

### 4.1.6 Callus proliferation

To find best medium for callus proliferation, creamish white nodular callus was shifted to MS medium supplemented with various combinations of auxins and cytokinins. Growth rate of callus was calculated by inoculating 2g of callus on the different media. MS medium supplemented with 2,4-D (14.61 µM) was found to be the best for callus proliferation wherein 8.62g callus was obtained from initial lump showing 4.31 increase in the fresh weight after 30 days. The callus was routinely maintained on MS medium supplemented with 2,4-D (14.61 µM) with regular subculturing done every 30 days to prevent the formation of mucilaginous callus.

**Table 4.1.4**- Effect of different media on callus proliferation

| S.No | Media combinations | Fresh weight of callus after 30 days (g) | Multiplication fold |
|---|---|---|---|
| 1 | MS+2,4-D(4.87 µM) | 3.08±0.037 | 1.54 |
| 2 | MS+2,4-D(9.74 µM) | 5.46±0.102 | 2.73 |
| 3 | MS+2,4-D(14.61 µM) | 8.62±0.135 | 4.31 |
| 4 | MS+2,4-D(9.74 µM)+BAP(2.26 µM) | 4.74±0.040 | 2.37 |
| 5 | MS+2,4-D(9.74 µM)+NAA(3.67 µM) | 5.14±0.060 | 2.57 |

### 4.1.7 Organogenetic differentiation from the callus:

#### 4.1.7.1 Rhizogenesis

The callus was found to be highly rhizogenic as numerous white roots differentiated from callus. Root differentiation occurred from calli on 2,4-D (14.61 µM) after 30 days of culturing in almost 100% of cultures. Initially a few roots were formed (Figure 4.1.21) but with further proliferation of the callus, more and more roots were organized (Figure 4.1.22-4.1.23) and covered the entire callus mass within 90 days. The roots were thick, white and had profuse root hairs.

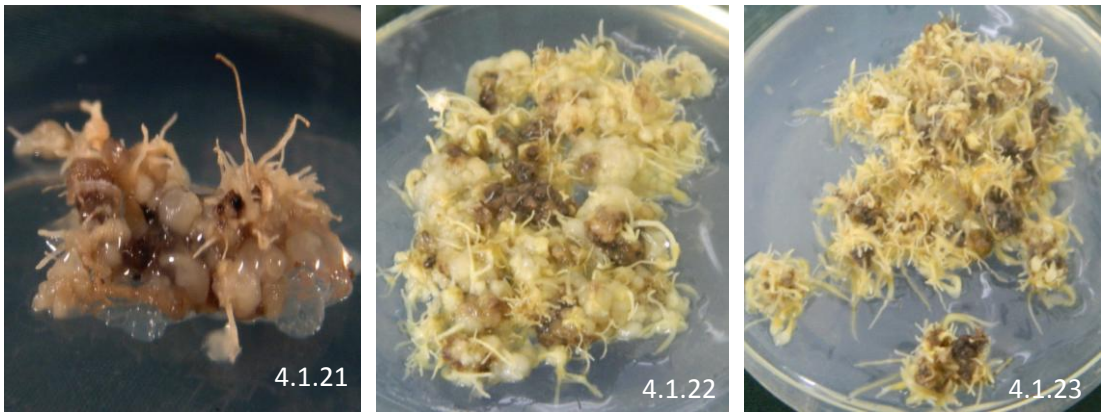



Figure 4.1.21- Emergence of roots from 30 days old callus; 4.1.22- Profuse rooting in 60 days old callus; 4.1.23- Profuse rooting which covered the entire callus mass after 90 days.

#### 4.1.7.2 Caulogenesis

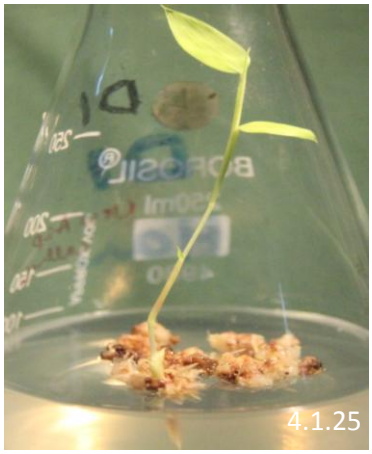
When the callus was shifted to MS medium supplemented with 2,4-D (9.74 µM) and BAP (1.13 µM), differentiation of shoots was observed. But the conversion frequency was very low. Only in 20% of the cultures, shoot differentiation was achieved as callus was observed to be highly rhizogenic. In the control, where only MS medium without PGRs was employed, no differentiation was observed and callus turned brown. Addition of any other cytokinin (Kn, TDZ, 2-ip) did not prove effective for inducing embryogenesis. Instead, they resulted in the browning of callus. Incorporation of any additional auxins (NAA, IBA, IAA) induced extensive rooting in

the callus. Addition of $GA_3$ or ABA into the medium did not influence embryoid formation. Their continuous presence resulted in the death of callus lumps. Figures 4.1.24-4.1.26 show 30 to 60 days old calli showing the formation of adventitious shoots.

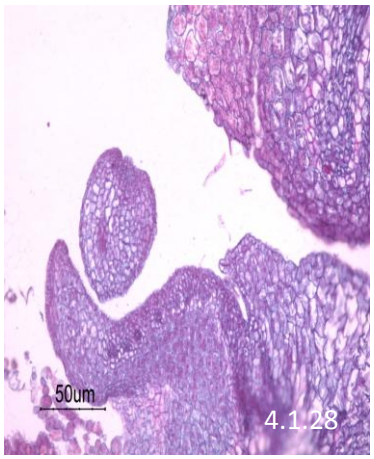
Histological investigation revealed the formation of globular meristemoids (Figure 4.1.28) from the callus after 45 days which developed into shoot bud initials (Figure 4.1.29). Figure 4.1.30 depicts a magnified shoot bud showing shoot apical meristem with subtending leaf primordia.

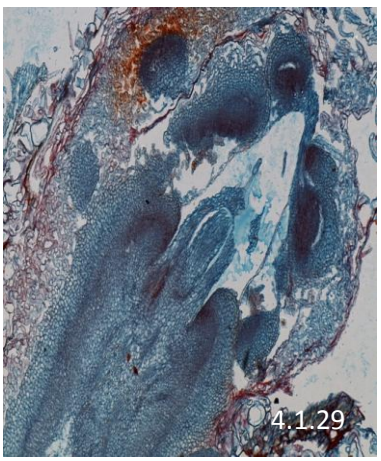
**4.1.7.3 Rooting and acclimatization**

The regenerated shoots were transferred to MS medium supplemented with IBA (14.76 µM) and NAA (3.67 µM) for inducing rooting. The complete plantlets with elongated shoot and root systems were formed after 45 days of culture. For acclimatization plantlets were transferred to plastic pots containing river bed sand and covered with plastic pots. Thereafter, they were shifted into potting mixture containing sand:soil:farmyard manure (1:1:1). The hardened plants were successfully transferred to the field conditions.



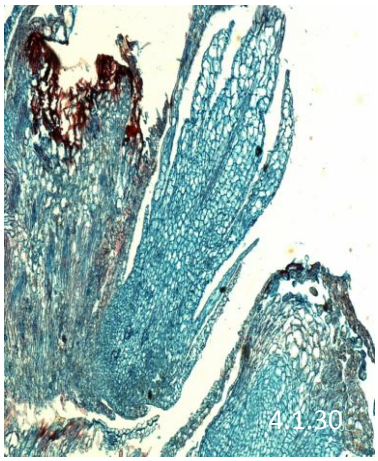




Figure 4.1.24- Shoot formation from callus on 2,4-D (9.74µM) and BAP (1.13µM) after 30 days of innoculation; Figure 4.1.25- Regeneration of shoots and roots from callus on 2,4-D (9.74µM) and BAP (1.13µM) after 45 days; Figure 4.1.26- Calli with numerous shoots and roots after 60 days of inoculation. Figure 4.1.27- Acclimatized plants. Figure 4.1.28- Meristemoid formation in the callus tissue; Figure 4.1.29- Formation of shoot bud initials in the callus tissue; Figure 4.1.30- Magnified view of a shoot bud.

## 4.2 Micropropagation studies in *Guadua angustifolia* Kunth

### 4.2.1 Initiation of aseptic cultures

#### 4.2.1.1 Choice of explants

For initiating aseptic cultures, various explants like apical bud, leaf and nodal explants were taken from 4-year-old potted plant. The explants were subjected to repeated washings after removal of leaf sheaths. This would remove all the adhering dust particles and microbes from the surface. The explants were then cleaned with a liquid detergent (Tween 20-HIMEDIA, Mumbai, India) followed by treatment with a suitable fungicide (e.g., Bavistin, 0.2%). Under sterile conditions in a laminar air flow bench, these explants were sterilized with 70% ethanol (v/v) for 1 minute and soaked in 0.04% (w/v) $HgCl_2$ for 6 minutes. Explants were inoculated onto MS medium supplemented with various adjuvants.

Apical buds of secondary and tertiary branches could not sustain growth in the culture medium and turned necrotic probably due to the physiological shock. The young leaf explants similarly did not show response in any of the media combination tried and turned necrotic after two weeks. Nodal explants from secondary and tertiary shoots proved most favourable for initiating aseptic cultures. These explants had an additional advantage of being available throughout the year. Moreover, they are easy to handle during sterilization.

#### 4.2.1.2 Position of explants

Position of explants had direct effect on culture initiation and quality of shoots formed under *in vitro* conditions. Explants taken from higher branches were found to respond better on multiplication medium with an early bud break.

#### 4.2.1.3 Bud break

Nodal explants cultured on MS medium without any growth regulator failed to respond. MS medium supplemented with BAP (8.86 μM) effectively induced sprouting in 90% of the explants within 15 days of inoculation. Bud break response declined with increase as well as decrease in the concentration of BAP. A high level of cytokinin is known to induce programmed cell death in cell cultures, yellowing of leaves and reduced root induction in the later stages (Carimi et al. 2003). Moreover, it often promotes ethylene biosynthesis in cultured tissues thus affecting the plant growth adversely (Abeles et al.1992). In kinetin supplemented MS medium, the explants failed to sprout.

#### 4.2.1.4 Standardization of multiplication medium

After bud break, sprouted buds were excised from nodal explants after 3 weeks of growth on initiation medium and cultured on MS medium supplemented with different concentrations and combinations of cytokinins. No shoot multiplication was observed on MS basal medium without growth regulators. However, shoot multiplication was observed in MS medium supplemented with different concentrations of BAP and 8.86 μM BAP gave maximum multiplication rate after 90 days (Figure 4.2.2). A significant increase in the shoot number was observed when BAP in conjunction with adenine sulfate was added to MS medium, thereby, establishing a definite synergism between these two growth regulators (Figure 4.2.3). MS liquid medium supplemented with BAP (8.86 μM) and adenine sulphate (13.5 μM) produced maximum lateral shoots with an average shoot number of 18.2 and shoot length of 7.06 cm after 90 days of culture. Addition of adenine sulphate to the culture medium can stimulate cell growth and greatly enhance shoot proliferation as it has a base structure similar to that of the cytokinins, hence shows cytokinin-like activity. In the

kinetin supplemented medium sprouted shoots failed to survive, hence its use was discontinued.

Table 4.2.1- Effect of various PGRs on multiple shoot proliferation in *G. angustifolia*

| S.No. | Media combinations | No.of shoots after 90 days | Length of longest shoot(cm) |
|---|---|---|---|
| 1 | MS+BAP( 4.43 µM) | 2.60±0.24 [f] | 4.46±0.05 [f] |
| 2 | MS+BAP( 8.86 µM) | 8.20±0.37 [c] | 5.76±0.08 [c] |
| 3 | MS+BAP(13.29 µM) | 7.80±0.20 [c] | 5.68±0.07 [c] |
| 4 | MS+BAP( 22.15µM) | 5.20±0.37 [d] | 5.10±0.07 [d] |
| 5 | MS+BAP(8.86 µM)+AdS(6.75 µM) | 14.00±0.31 [b] | 6.84±0.06 [b] |
| 6 | MS+BAP(8.86 µM)+AdS(13.5 µM) | 18.20±0.20 [a] | 7.06±0.08 [a] |
| 7 | MS+Kn( 2.32 µM) | 0.00±0.00 [g] | - |
| 8 | MS+Kn(4.65 µM) | 0.00±0.00 [g] | - |
| 9 | MS+BAP(8.86 µM)+Kn(1.16 µM) | 5.60±0.24 [d] | 4.86±0.05 [e] |
| 10 | MS+BAP(8.86 µM)+NAA(1.83 µM) | 3.60±0.40 [e] | 3.30±0.09 [g] |

Duncan test; p=0.05; Values followed by the same letters in superscript within the column are not significantly different.

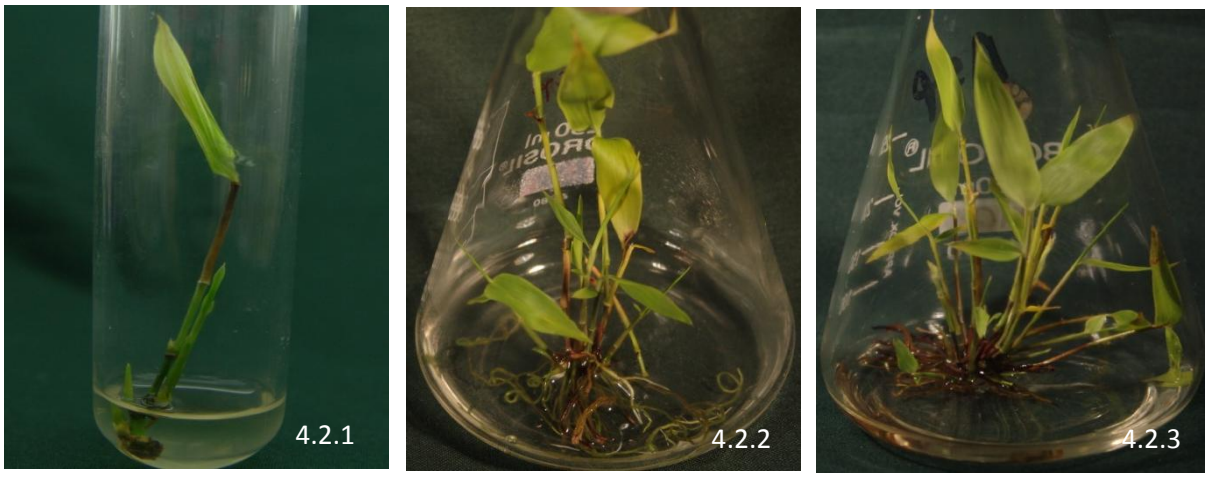
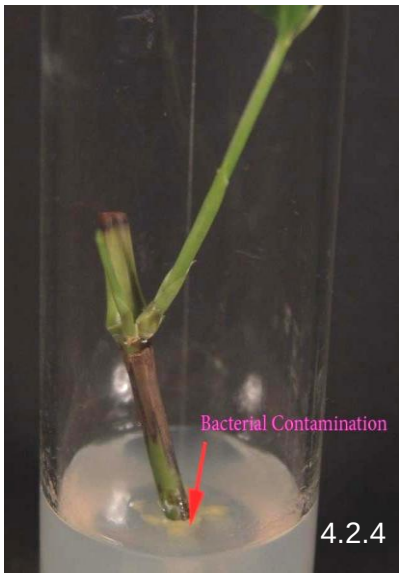



Figure 4.2.1-Induction of multiple shoots in *G.angustifolia* after 15 days; 4.2.2- Multiple shoot formation on MS+BAP (8.86 µM) after 90 days; 4.2.3- Multiple shoot proliferation on liquid MS medium supplemented with BAP (8.86 µM) and AdS (13.5 µM) after 90 days.

### 4.2.2 Bacterial contamination




Our attempts to obtain aseptic *in vitro* cultures using explants of *Guadua* from potted plant and to optimize a micropropagation procedure were hindered by persistent appearance of bacterial contamination in the cultures. Bacterial growth appeared as a cloudy zone in the agar medium around the shoot base within 20 days, invariably in all the cultures (Figure-4.2.4) and hindered growth and multiplication of cultures. Bacterial contamination in tissue culture is well documented and the failure of surface sterilization procedures to produce aseptic cultures is a major problem with woody plants. The growth medium selected for *in vitro* propagation also serves as a good source of nutrients for microbial growth. These microbes further compete adversely with plants for nutrients.

#### 4.2.2.1 Isolation and identification of bacteria

Bacterial growth appeared as a cloudy zone in the agar medium around the shoot base within 20 days invariably in all the cultures. The contaminating bacteria were isolated by picking material with a loop from visibly contaminated culture and streaking directly on the LB medium. After incubation at 25$^{o}$C for 24 hours, two types of colonies were observed. Pure cultures of these bacteria were obtained by picking up the colonies and streaking them onto the fresh medium. These cultures were further maintained in glycerol stock at –80$^{o}$C.

16S rRNA gene sequence analysis was utilized to identify bacterial contaminants in *Guadua angustifolia* Kunth**.** These contaminants were found to be highly similar to

*Pantoea agglomerans* (NCBI # SR872702) and *Pantoea ananatis* (NCBI # SR872704). Both bacteria are gram negative and closely related.

#### 4.2.2.2 Antibiotic treatment of plants

The luria agar plates containing different antibiotics like kanamycin, carbenicillin, ampicillin, rifampicin, and streptomycin sulfate were inoculated with isolated bacterial contaminants for antibiotic screening.

On the basis of their effectiveness in antibiotic sensitivity testing, two antibiotics namely kanamycin and streptomycin sulfate were selected. To test their effectiveness in eliminating bacterial contamination, the antibiotics were added to the multiplication medium, i.e., the liquid MS medium containing BAP (8.86μM) and adenine sulfate (13.5 μM) in the following dosages: 0, 5, 10, 15, 25, 40, 50 μg/ml either alone and in combinations with each other. Contaminated explants were then dipped in this medium for 10 days. Controls (plant tissue grown in the multiplication medium without antibiotics) were also included with each experiment. After 10 days of the antibiotic treatment, the physical condition of plants was noted again and then placed in the liquid multiplication medium without any antibiotic. Shoots with no detectable signs of bacterial contamination were individually transferred onto the fresh medium without antibiotics and subcultured every 3 weeks. Growth rate and plant appearance were monitored to determine whether the antibiotics had any phytotoxic effects on plants during the multiplication and rooting phase.

Antibiotic sensitivity test revealed kanamycin and streptomycin sulfate as the most effective antibiotic against the contaminating bacteria (Table 4.2.2). The kanamycin was least phytotoxic during micropropogation of *G. angustifolia*. The shoot tips were grown for 10 days on the multiplication medium containing the kanamycin (10 μg/ml). The addition of kanamycin grossly inhibited the bacterial growth while allowing the

formation of high-quality *Guadua* shoots (Figure 4.2.7). In contrast, streptomycin was effective in reducing bacterial growth in tissue culture at higher concentrations (15 μg/ml). Moreover, the shoot number and the quality of shoots were also reduced. Kanamycin interacts with the 30S subunit of prokaryotic ribosomes. It induces substantial amount of mistranslation and indirectly inhibits translation during protein synthesis. Streptomycin binds to the S12 protein of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This prevents initiation of protein synthesis and leads to death of microbial cells. It may also inhibit protein synthesis in chloroplasts and mitochondria in plant tissues and thus resulting in small and yellow leaves.

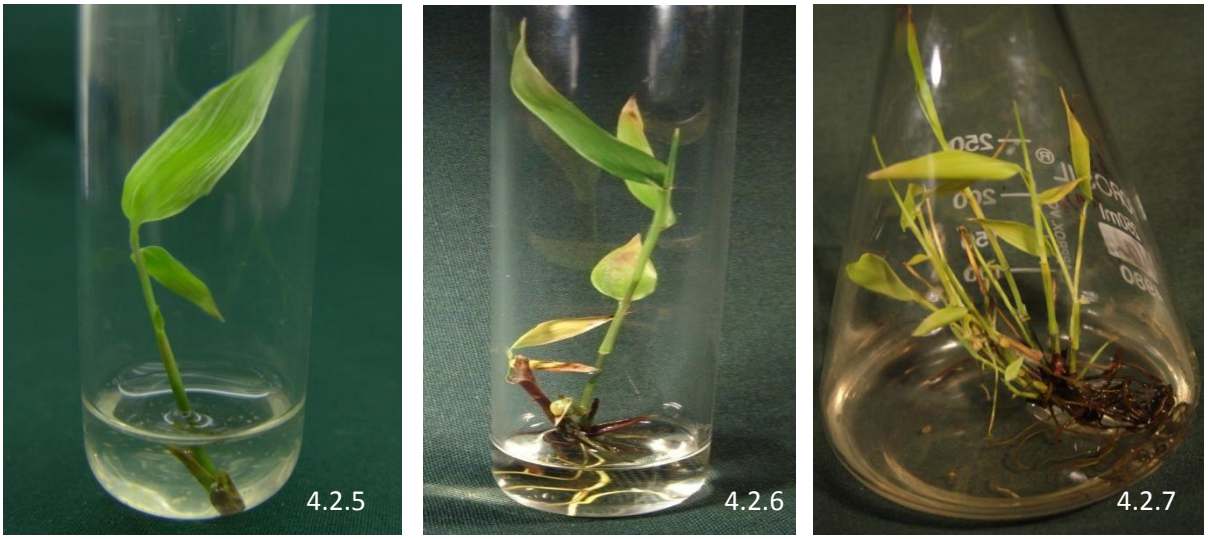



Figure 4.2.5- Treatment of infected shoot in multiplication medium containing kanamycin (10 μg/ml); 4.2.6- Initiation of lateral shoots after kanamycin treatment; 4.2.7- Formation of healthy shoots after kanamycin treatment.

Table 4.2.2- The effect of various antibiotics on the growth of bacteria and *Guadua angustifolia* Kunth.

| **Antibiotic** | **Concentration (μg/ml)** | **Removal of bacteria** | **Health status of shoots** |
|---|---|---|---|
| Streptomycin sulfate | 0 | – | Overgrowth of bacteria inhibited shoot survival |
| | 5 | + | Overgrowth of bacteria inhibited shoot survival |
| | 10 | ++ | Shoots were fresh, green, and healthy |
| | 15 | +++ | Shoots were yellowish green with small leaves |
| | 25 | +++ | Shoot necrosis |
| | 40 | +++ | Shoot necrosis |
| | 50 | +++ | Shoot necrosis |
| Kanamycin | 0 | – | Over growth of bacteria inhibited shoot survival |
| | 5 | ++ | Overgrowth of bacteria inhibited shoot survival |
| | 10 | +++ | Shoots were fresh, healthy, and green |
| | 15 | +++ | Shoots were yellowish green |
| | 25 | +++ | Shoot necrosis |
| | 40 | +++ | Shoot necrosis |
| | 50 | +++ | Shoot necrosis |

"+" sign denotes a positive response to some extent; "++" sign denotes a positive response to moderate extent; "+++" sign denotes a complete positive response; "–" sign denotes no response

### 4.2.3 Plant multiplication

When the original clump had produced enough lateral shoots, it was divided into the groups of 3-5 shoots which were planted on the multiplication medium. A constant multiplication rate of 2.5 was observed after 45 days. Single shoot used as propagule failed to multiply on the multiplication medium.

### 4.2.4 Rooting

Rooting was obtained simultaneously during shoot multiplication on the multiplication medium without addition of any auxin in 100% of the cultures. From approximately 45 days after culture initiation, a continuous and exponential development of new roots was observed. In fact, rooting was so vigorous that it inhibited the shoot proliferation. So roots had to be trimmed off during subculture to enhance shoot multiplication.

### 4.2.5 Acclimatization

Well developed and rooted clumps of 5-6 shoots were selected for hardening (Figure 4.2.8). Plants were initially shifted to the plastic pots containing soil and were covered with jars for maintaining high humidity for 30 days (Figure 4.2.9). These were then shifted to a mixture of sand:soil:manure (1:1:1) and kept in a green house. They were regularly sprayed with Hoagland solution after seven days interval. Almost 100% of the plants survived during acclimatization (Figure 4.2.10).




Figure 4.2.8- Well developed rooted plants of *Guadua*; 4.2.9- *Guadua* plants in pots containing river bed soil for hardening under green house conditions; 4.2.10- Acclimatized plants of *G. angustifolia.*

## 4.3 Micropropagation studies in *Phyllostachys pubescens* Mazel ex H. De Lehale

### 4.3.1 Initiation of aseptic cultures

#### 4.3.1.1 Nodal explants

Nodal explants were taken from two year old field raised elite mother plant. After initial surface sterilization treatment with bavistin (0.1%) and streptomycin sulfate (0.4%) for 20 minutes, explants were subjected to treatment with 70% alcohol followed by 0.04% $HgCl_2$ for 7 minutes in a laminar air flow bench before they were inoculated on MS basal medium for inducing bud sprouting. On MS basal medium only, 10% of the explants sprouted after 30 days. However, with the addition of BAP (13.29 µM) in the medium sprouting was achieved much earlier (Figure 4.3.1). Highest sprouting rate was obtained on 3mg/l BAP and an increase or decrease in the concentration of BAP caused a decline in bud sprouting. It was further observed that the sprouting was achieved only in the explants collected during winters in the months of December to February. Nodal explants taken during March-November failed to sprout. Even incorporation of $GA_3$ proved ineffective in this case.


#### 4.3.1.2 Shoot multiplication

Sprouted buds were excised from the nodal explants and inoculated onto MS medium supplemented with different concentrations and combination of cytokinins for inducing shoot proliferation. But none of the combinations tried so far could induce shoot proliferation. Conjunction of cytokinins with auxins also failed to induce

any response. The sprouts remained alive for 4 weeks on medium containing 2-ip but growth was not sustained thereafter.

Due to failure to induce multiple shoot proliferation from nodal explants taken from mature field raised plant, experiments were further carried out using nodal explants from *in vitro* raised seedlings.

#### 4.3.1.3 Raising aseptic seedlings

Seeds obtained from China were used to raise aseptic seedlings. After dehusking, the healthy looking seeds were selected, soaked overnight and subjected to surface sterilization treatment. Initially, 90% of the seeds germinated within 10-12 days on basal MS medium. However, the seeds stored for 5 months at $0^{o}$C showed only 60% germination and the germination rate declined further with increase in the storage period. On a hormone free MS medium, generally a single unbranched shoot developed from the seed. Incorporation of BAP neither affected the percentage of germination nor resulted in the formation of more shoots. Addition of kinetin inhibited germination of seed.

#### 4.3.1.4 Contamination

Fungal contamination was the major bottleneck during seed germination. The survival percentage of *in vitro* raised seedlings was 40%. To counter fungal contamination, seeds were soaked in 1% bavistin solution and kept on a shaker for 12 hours. This appreciably reduced fungal contamination to 30%.

### 4.3.2 Shoot proliferation

Nodal explants (2 cm) were excised from one month old *in vitro* raised seedlings and were inoculated onto different concentrations and combinations of PGRs. On MS basal medium, the single unbranched shoot formed remained green for about 7 days but thereafter signs of necrosis appeared and it died within 15 days without showing

any notable growth. Incorporation of BAP (2.26-22.15 μM) did not result in multiple shoot formation. Addition of kinetin also proved ineffective. When nodal explants were planted on MS medium supplemented with TDZ, nodal bud break was observed after 15 days (Figure-4.3.3). Initial treatment with TDZ (0.9 μM) and Kn (4.65 μM) for 30 days followed by their subculture to MS basal medium was found to be effective for inducing multiple shoots and their elongation. On an average, 8.6 shoots were obtained after 8 weeks on MS basal medium after initial treatment with TDZ and Kn (Figures 4.3.6, 4.3.7; Table 4.3.1). Prolonged exposure for more than 30 days to TDZ inhibited the proliferation of shoots and also resulted in the formation of stunted shoots. Addition of BAP to the TDZ supplemented medium lowered the multiplication rate achieved with TDZ alone. However, an increase in the concentration of TDZ (1.35-2.25 μM) inhibited shoot proliferation.

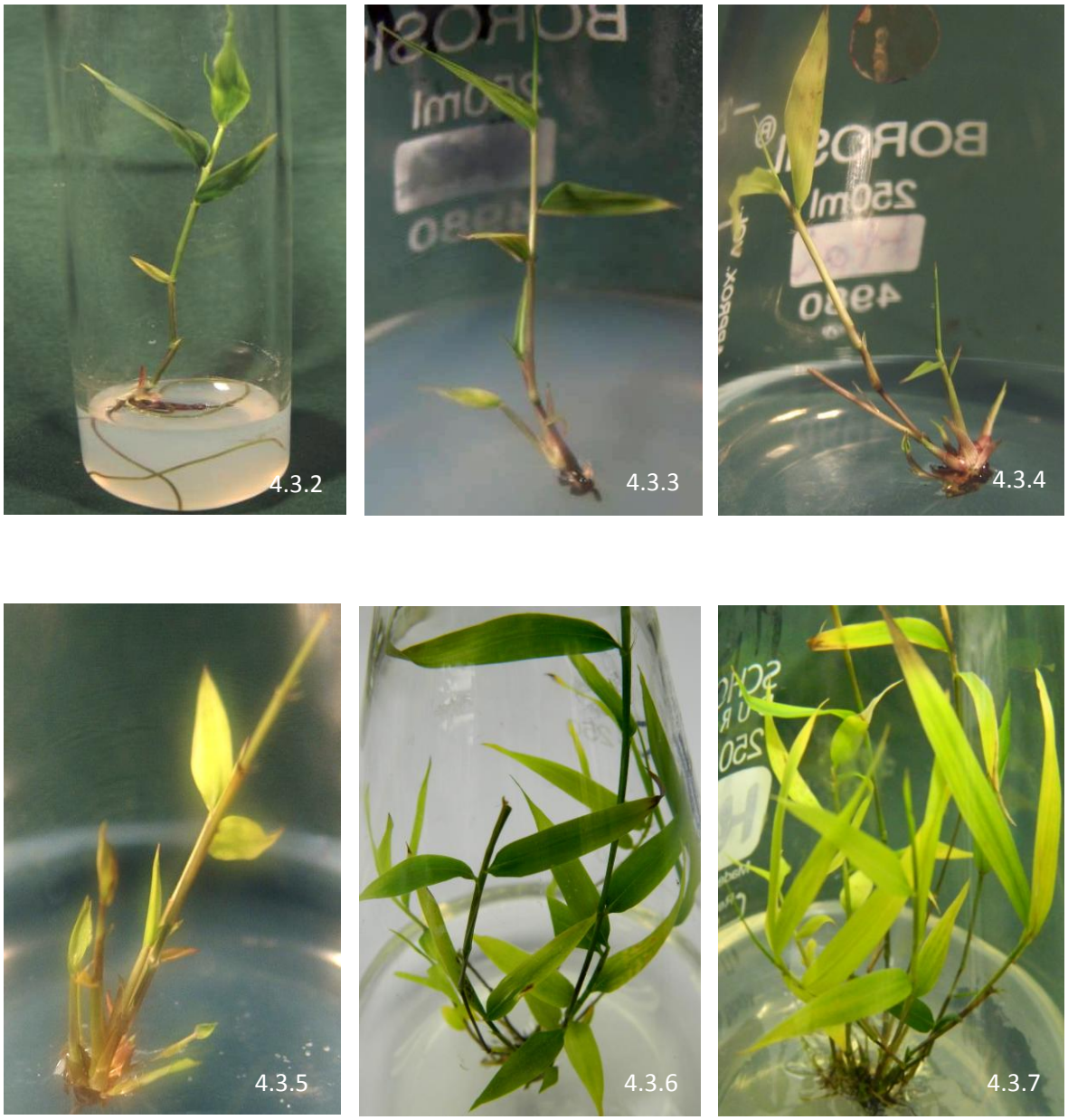



Figure 4.3.2- 40-days-old seedlings of *P. pubescens* on basal MS medium; Figure 4.3.3- Axillary bud break on TDZ (0.9 µM) supplemented medium; Figure 4.3.4- Shoots (5-6) formed on MS basal medium after initial treatment with TDZ (0.9 µM); Figure 4.3.5- Shoots initiation on MS medium supplemented with TDZ (0.9 µM) and Kn (4.65 µM); Figures 4.3.6-4.3.7 Shoot proliferation on MS medium after 6 weeks and 8 weeks of culture respectively.

Table 4.3.1- Effect of concentration and combination of cytokinins on shoot multiplication in *Phyllostachys pubescens.*

| S.No | Treatments (30 days) | Number of shoots | Length of shoots (cm) |
|---|---|---|---|
| 1 | TDZ ( 0.25 µM) | 2.40±0.24 [f] | 2.94±0.10 [f] |
| 2 | TDZ (0.45 µM) | 3.40±0.24 [e] | 3.16±0.05 [e] |
| 3 | TDZ ( 0.9 µM) | 5.00±0.31 [d] | 4.26±0.08 [d] |
| 4 | TDZ ( 0.45 µM)+Kn(4.65 µM) | 6.80±0.37 [b] | 4.36±0.08 [d] |
| 5 | TDZ ( 0.9 µM)+Kn(4.65 µM) | 8.60±0.24 [a] | 5.24±0.05 [b] |
| 6 | TDZ (2.25 µM)+Kn(4.65 µM) | 6.00±0.31 [c] | 4.96±0.05 [c] |
| 7 | TDZ (0.9 µM)+Kn(9.3 µM) | 5.40 ±0.24 [c,d] | 5.42±0.03 [a] |
| 8 | TDZ (0.9 µM)+BAP(4.43 µM) | 0.00±0.00 [g] | - |
| 9 | BAP (4.43 µM) | 0.00±0.00 [g] | - |

Duncan test; p=0.05; values followed by the same letters in superscript within the column are not significantly different.

Addition of charcoal to the multiplication medium proved inhibitory for shoot multiplication. After the optimization of phytohormones for shoot proliferation, the effect of various gelling agent, such as agar (0.8%) and phytagel (0.25%) was also studied. In the present study, replacement of solidified medium with liquid medium did not improve proliferation rate significantly. Use of phytagel instead of agar was found to be beneficial for promoting shoot multiplication. In agar gelled medium, shoots remained dwarf and failed to elongate.

#### 4.3.2.1 Effect of bud position on proliferation efficiency

In order to find out the suitability of different nodes for their proliferation efficiency, nodes from the elongated shoots raised *'in vitro'* were numbered from bottom and after excision transferred to MS medium supplemented with TDZ and Kn. Lowest node showed maximum efficiency to proliferate (Figure 4.3.8). About 70% nodes proliferated within one month while proliferation ability steadily declined with position of the nodes from bottom to top.

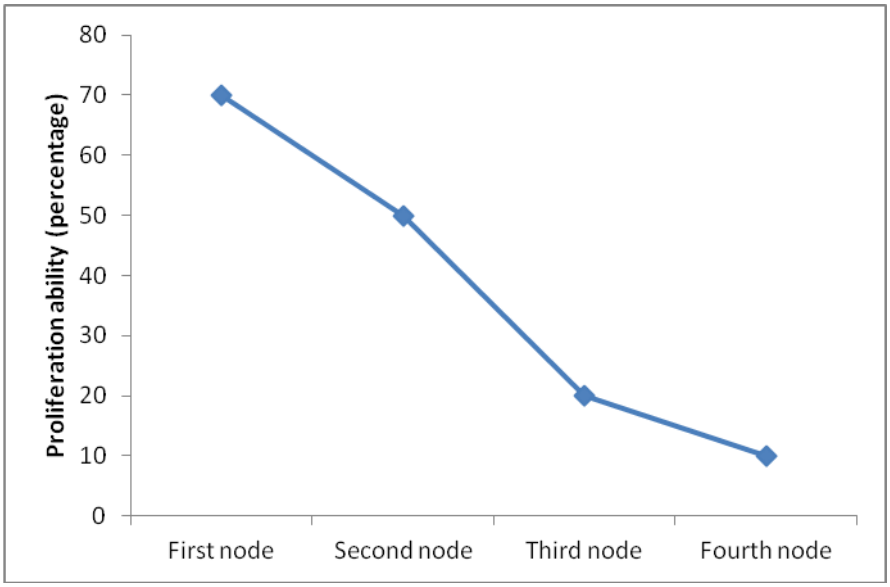



Figure 4.3.8- Effect of position of bud on proliferation ability

#### 4.3.2.2 Effect of carbon source on multiplication rate

To study the effect of different carbon sources on shoot multiplication, 3 shoots per propagule were cultured on MS medium containing TDZ and Kn and supplemented with either 3% sucrose, glucose or table sugar respectively. Glucose was found to be most suitable carbon source as 7.6 shoots were obtained after 4 weeks of culture, while 5.6 shoots were obtained on sucrose supplemented medium (Figure 4.3.9).

Three percent glucose proved to be ideal for shoot multiplication both in terms of number as well as their overall growth. When 2% glucose was added to the medium,

the number of new shoots formed declined and shoots did not look healthy. At increased levels of glucose (4% and 5%) shoots turned necrotic.

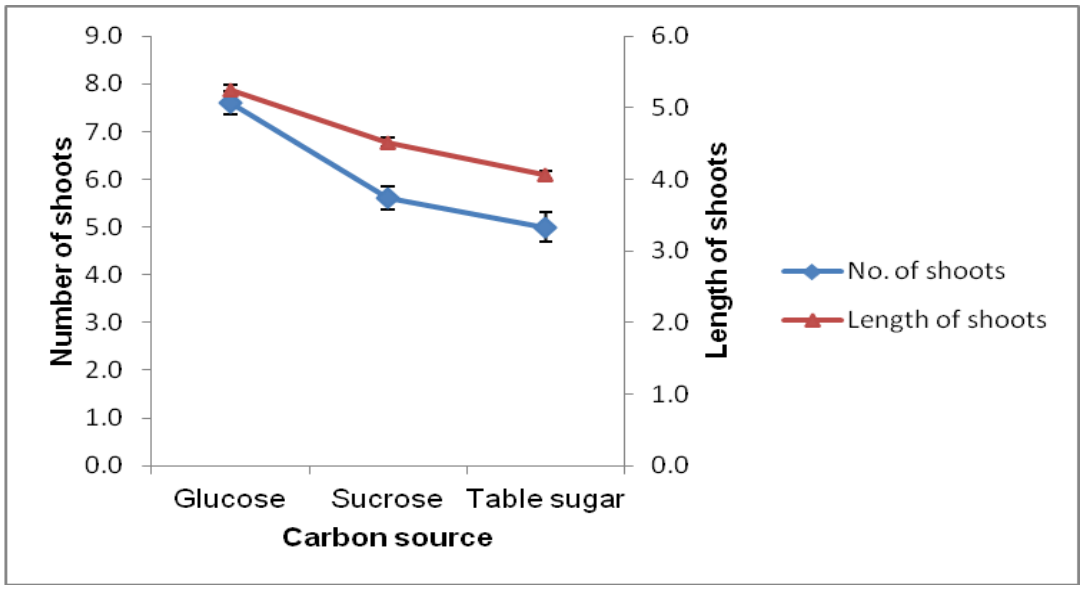



Figure 4.3.9- Effect of carbon source on shoot multiplication

### 4.3.3 Rooting

The clumps of 3 to 4 shoots (>3.0 cm) were inoculated onto different media for inducing rooting. Of the three basal media (MS, B5 and Woody Plant Medium) tried, MS medium proved best in term of rooting percentage and general condition of the plants during rooting of shoots. Since *in vitro* raised shoots failed to root on a hormone free basal medium, use of various auxins was attempted.

Microshoots were rooted on MS medium following their initial incubation for 15 days on medium containing IBA. Initial exposure to IBA (9.84 µM) and subsequent withdrawal of auxin from the medium was found to be very effective for overall root development. This two step procedure resulted in root induction in almost 75% of cultures within 4 weeks of transfer to auxin free medium (Table 4.3.2). Generally 2-3 roots emerged from the basal end of the propagule (Figure 4.3.11-4.3.12). Prolonged treatment in auxin supplemented medium for more than 15 days resulted in necrosis

of shoots and inhibition of rooting. No rooting was induced when IAA or NAA alone were used at various concentrations.

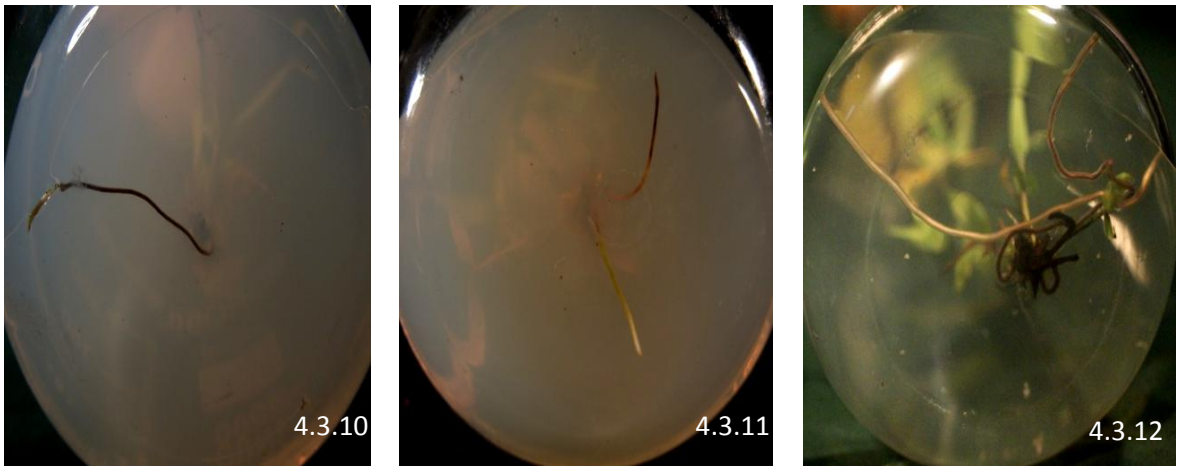



Figure 4.3.8- Root formation after initial exposure to IBA (4.92 µM); 4.3.9- Root formation on MS basal medium after pulse treatment with IBA (9.84 µM) after 2 weeks; 4.3.10-Root formation after 4 weeks.

Table 4.3.2- Effect of different auxins on rooting.

| S.No | Treatments (15 days) | Number of roots | Percentage rooting |
|---|---|---|---|
| 1 | IBA (4.92 µM) | 1.80±0.20 [b] | 46 |
| 2 | IBA (9.84 µM) | 2.80±0.20 [a] | 75 |
| 3 | IBA (14.76 µM) | 2.40±0.24 [a] | 64 |
| 4 | NAA (7.35 µM) | 0.00±0.00 [c] | 0 |
| 5 | IAA (5.71µM) | 0.00±0.00 [c] | 0 |
| 6 | IBA (4.92 µM)+NAA (3.67 µM) | 0.00±0.00 [c] | 0 |

Duncan test; p=0.05; values followed by the same letters in superscript within the column are not significantly different.

### 4.3.4 Transplantation

Four week old rooted plantlets with 4-5 shoots bearing 6-8 leaves were used for transplantation (Figure 4.3.13). Only 30% of the rooted plants directly transferred to the potting mixture (riverbed sand:soil:manure:: 1:1:1) survived. The surviving plants took about 8 weeks to develop new shoots. In contrast, 80% survival was achieved when the plantlets were initially shifted to the pots containing river bed sand and were covered with transparent jars for maintaining high relative humidity for 4 weeks followed by their transfer to the potting mixture containing riverbed sand:soil:manure in 1:1:1 ratio (Figure 4.3.14). During hardening, lamina of the leaves expanded significantly. Also, the leaves turned greener and healthier. Moreover, new shoot were formed within 4 weeks of shifting. After hardening these plants were kept in a polytunnel for six months (Figure 4.3.15). These were then transplanted in the field in the pits (2ft×2ft×2ft) and filled with well rotten farm yard manure (Figure 4.3.16 a,b,c). The plant to plant distance was kept at 6 meters and the growth data was recorded.







Figure 4.3.13-Rooted plants before hardening; 4.3.14-Hardened plants of *P. pubescens after* 2 months of shifting to potting mixture; 4.3.15- Six month old hardened plants; 4.3.16 a,b,c- Well established plants of *P. pubescens* in field.

## 4.4 Micropropagation studies in *Bambusa tulda* Roxburgh

### 4.4.1 Initiation of aseptic cultures

#### 4.4.1.1 Explant

Different explants like nodal segments from secondary and tertiary branches, leaves and young buds excised from secondary branches were tested for their suitability for regeneration potential. But only nodal segments were found to be suitable for culture initiation. Leaf explants failed to respond on any of the media combinations tried while young buds from main culm failed to germinate and proliferate (Figure 4.4.1).

Single node cuttings from second and third internode of secondary branches of a 12-year old vegetatively propagated plant of *B .tulda* were collected and used for initiating *in vitro* cultures. Prior to surface sterilization, leaf sheath that envelopes the axillary bud and a part of the upper internode was removed. Fungal contamination was found to be the major bottle neck in initiating aseptic cultures, so a suitable sterilization procedure was adopted involving use of bavistin (0.2%) along with blitox (0.1%) for 20 minutes followed by treatment with Sodium hypochlorite (2%) for 6-8 minutes. Under sterile conditions, the explants were treated with $HgCl_2$ before inoculating on to MS medium for sprouting.



Figure 4.4.1- Young bud excised from main culm; 4.4.2- Nodal explant from secondary branch of 12 -year-old *B. tulda.*

#### 4.4.1.2 Season vs Culture initiation

The collection time of explant for culture initiation was found to play a significant role in increased bud break and number of shoots produced per explant. Collection of explants during spring in the months of March and April gave better response in terms of increased bud break, early shoot initiation and decreased contamination. Furthermore, these shoots kept on multiplying during subsequent subculturing. The explants collected during other months failed to show sustainable development as the shoots failed to multiply and survive after third subculturing. Even incorporation of phytohormones or additional growth adjuvants into the medium could not induce proliferation and survival of these shoots. During rainy season (June-August) maximum contamination (100%) was observed. Hence, these months were found to be unsuitable for initiation of cultures and bud break. Variation in bud break percentage in *B. tulda* during the year is shown in the figure 4.4.3.

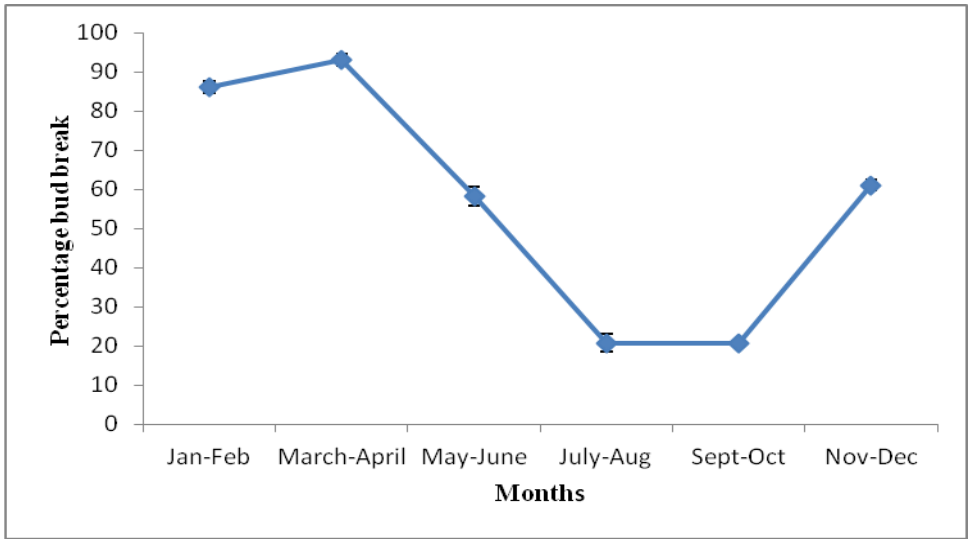



Figure 4.4.3- Effect of season on percentage bud break in *B. tulda*

#### 4.4.1.3 Position of node

Position of explant on the mother clump was found to affect the response in terms of bud break and the number of shoots formed. Single node cuttings from second and third internode of secondary branches were observed to sprout early when inoculated. Moreover, each of these buds sprouted into clump of 3-4 shoots. When these sprouts were cultured on the multiplication medium, their multiplication rate was relatively faster. Whereas, the nodal explant collected from tertiary branches sprouted into single shoot which remained green in the culture medium for about 15 days, but failed to multiply further and turned necrotic and eventually died.

### 4.4.2 Establishment of multiple shoots

Nodal explants cultured on MS basal medium without any growth regulators failed to sprout. MS medium supplemented with BAP (8.86 µM) was found to induce sprouting within 15 days. An increase in concentration of BAP considerably reduced the percent bud break. After 25 days of incubation of nodal explants, the sprouted buds were excised from the mother stumps and placed on cytokinins supplemented

liquid MS medium for shoot proliferation. Since the sprouts remained dormant and eventually turned necrotic on MS basal medium, this necessitated the inclusion of cytokinins in the shoot multiplication medium either alone or in combination. Of the different concentrations of BAP tested, 13.29 µM BAP gave the best proliferation rate where 6.66 shoots were obtained (Figure 4.4.5). Higher concentration of BAP not only lowered the multiplication rate but also resulted in stunted shoots. Kinetin didnot result in shoot proliferation when added alone at concentrations ranging from 2.32 µM to 9.3 µM: the shoots remained dormant for some time and ultimately died. However, Kn (4.65 µM) when used with the optimal concentration of BAP (8.86 µM) enhanced the multiplication of shoots, on an average 21.66 shoots were formed after 60 days (Figure 4.46). Initially little response was observed during 40-45 days of incubation of the excised buds on the multiplication medium, however, during subsequent subculturing shoot proliferation initiated and the frequency of multiplication gradually increased with time. Table 4.4.1 shows the effect of different concentrations and combinations of cytokinins on multiple shoot proliferation in *B. tulda.*

Table 4.4.1- Number of shoots obtained on MS medium containing different concentrations and combinations of BAP, Kn and adenine sulfate after 45 and 60 days.

| S.No | Treatment | No. of shoots after 45 days | No. of shoots after 60 days |
|---|---|---|---|
| 1 | BAP(4.43 µM) | 3.33±0.33 $^{kl}$ | 4.33±0.33 $^{jk}$ |
| 2 | BAP(8.86 µM) | 4.66±0.33 $^{jk}$ | 6.33±0.33 $^{ghi}$ |
| 3 | BAP(13.29 µM) | 5.00±0.00 $^{ij}$ | 6.66±0.33 $^{gh}$ |
| 4 | BAP(22.15 µM) | 2.66±0.33 $^{l}$ | 0.00±0.00 $^{m}$ |
| 5 | BAP(8.86 µM)+K(1.16 µM) | 7.33±0.33 $^{g}$ | 13.33±0.66 $^{d}$ |
| 6 | BAP(8.86 µM)+K(2.32 µM) | 7.66±0.33 $^{g}$ | 14.66±0.33 $^{c}$ |
| 7 | BAP(8.86 µM)+K(4.65 µM) | 10.66±0.66 $^{e}$ | 21.66±0.88 $^{a}$ |
| 8 | BAP(13.29 µM)+K(2.32 µM) | 5.33±0.33 $^{hij}$ | 9.33±0.66 $^{f}$ |
| 9 | BAP(8.86 µM)+K(9.3 µM) | 11.00±0.57 $^{e}$ | 20.33±0.33 $^{b}$ |
| 10 | BAP(8.86 µM)+AS(2.7 µM) | 4.66±0.33 $^{jk}$ | 6.66±0.33 $^{gh}$ |
| 11 | BAP(8.86 µM)+AS(5.4 µM) | 4.66±0.33 $^{jk}$ | 6.33±0.33 $^{ghi}$ |
| 12 | BAP(8.86 µM)+AS(8.1 µM) | 4.00±0.57 $^{jkl}$ | 4.66±0.66 $^{jk}$ |

Duncan test; p=0.05; Values followed by the same letters in superscript within the column are not significantly different

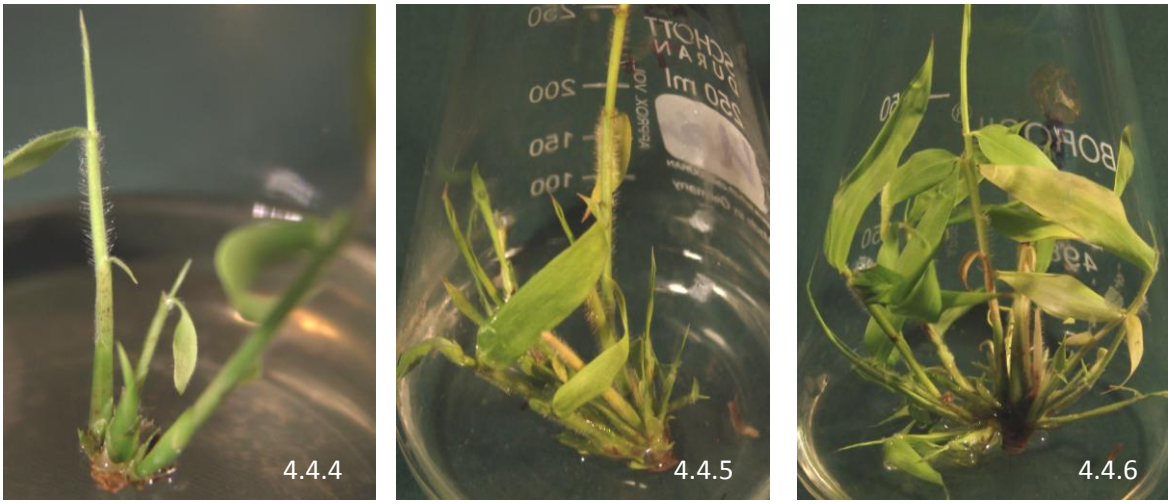



Figure 4.4.4-Initiation of multiple shoots in *B. tulda*; 4.4.5- Formation of multiple shoots on BAP (13.29 µM) after 60 days; 4.4.6- Shoot proliferation on liquid MS medium supplemented with BAP (8.86 µM) and Kn (4.65 µM) after 60 days.

Once the multiplication medium was standardized, initial shoot clumps were divided into clump of 5-6 shoots each (called as a propagule) and were cultured on multiplication medium. Popagules containing a minimum of 5-6 shoots proliferated at a maximum rate whereas single shoots failed to proliferate and survive during subculture.

#### 4.4.2.1 Effect of gelling agent

Addition of different gelling agents like agar or gelrite to the shoot multiplication medium containing BAP (8.86 µM) and Kn (4.65 µM) considerably reduced the multiplication rate, clearly indicating that the liquid medium was best suited for shoot multiplication in this species.

#### 4.4.2.2 Carbon source

Among the different carbon sources (sucrose, table sugar and glucose) tested, sucrose was found to be the most suitable as maximum multiplication rate was achieved on it. On the other hand, on glucose fortified medium, the least multiplication rate was achieved. Moreover, shoots failed to survive after second subculture cycle.

Two percent sucrose proved to be ideal for shoot multiplication both in terms of number of shoots as well as their overall growth. At 1% sucrose, the number of new shoots declined and shoots did not look healthy. Increased levels of sucrose (3%) did not affect shoot number and the shoots turned necrotic and ultimately died.

#### 4.4.2.3 Propagule

Multiplication fold was observed to be directly influenced by number of shoots used as propagule for initiating cultures. When the original clump was divided into clumps of 3,6 and 9 shoots and cultured onto multiplication medium containing BAP (8.86 µM) and Kn (4.65 µM), 6 shoots per propagule were found to be most effective for

multiplication of shoots. Single shoot used as propagule remained green for 2 weeks but failed to survive thereafter.

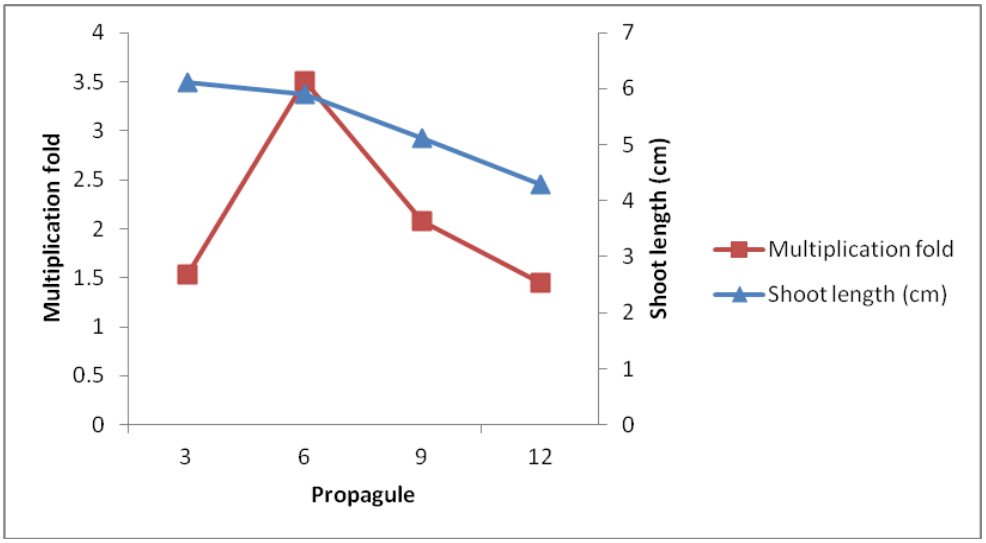



Figure 4.4.7- Effect of the number/clump on shoot multiplication in *B. tulda*

### 4.4.3 Rooting

Rooting is the major bottleneck during micropropagation of bamboos in general. Clusters of three to four shoots obtained after completion of shoot multiplication cycle were transferred to various concentrations and combinations of auxins (IBA, IAA and NAA). But none of these auxins could induce rooting. Two step treatment by initial exposure of shoots to higher concentrations of IBA, NAA and IAA followed by their shifting to PGR free medium also proved ineffective for root induction. Even addition of coumarin (6.84-68.4 µM) into the medium failed to induce rooting. Attempts were made to induce rooting *ex vitro* by treating shoots with high concentration of auxins followed by their transfer to pots containing sand but rooting could not be induced under any of the treatments tried.

### 4.4.4 Callus induction

New sprouts obtained from *in vivo* nodal segments were inoculated on MS medium supplemented with 2,4-D and NAA for callus induction. But no callusing could be induced in them. So *in vitro* raised shoots were used and nodal segments were excised and cut into small pieces before inoculating onto 2,4-D or NAA fortified medium. Callus could only be induced on higher concentration of 2,4-D (29.22 µM) when used in conjuction with coconut milk. On MS medium supplemented with 2,4-D (29.22 µM) and coconut milk (10%), two types of calli were obtained 1) Nodular and compact creamish white callus (Figure-4.4.9) 2) Mucilagenous and translucent callus (Figure-4.4.10). Mucilagenous callus was discarded because of its failure to respond for any regeneration or differentiation, only nodular callus was retained. Callus induction started within 10 days of inoculation at cut ends of the nodal explant (Figure 4.4.8 ) and within 2 months the entire explant turned into a mass of callus. Incorporation of NAA or BAP to the callus induction medium was found to be inhibitory.

Table 4.4.2- Effect of different concentrations of 2,4-D together with coconut milk (CM) on callus induction from nodal explants of *in vitro* raised shoots

| S.No. | Media composition | Percent callus induction | Days required |
|---|---|---|---|
| 1 | MS | 0 | - |
| 2 | MS+NAA(14.7 µM) | 0 | - |
| 3 | MS+2,4-D(4.87 µM)+CM(10%) | 15 | 30 |
| 4 | MS+2,4-D(14.61 µM)+CM(10%) | 32 | 25 |
| 5 | MS+2,4-D(29.22 µM)+CM(10%) | 68 | 10 |
| 6 | MS+2,4-D(38.96 µM)+CM(10%) | 64 | 10 |
| 7 | MS+2,4-D(29.22 µM)+BAP(2.26µM) | 0 | - |
| 8 | MS+2,4-D (29.22 µM)+NAA(3.67µM) | 0 | - |

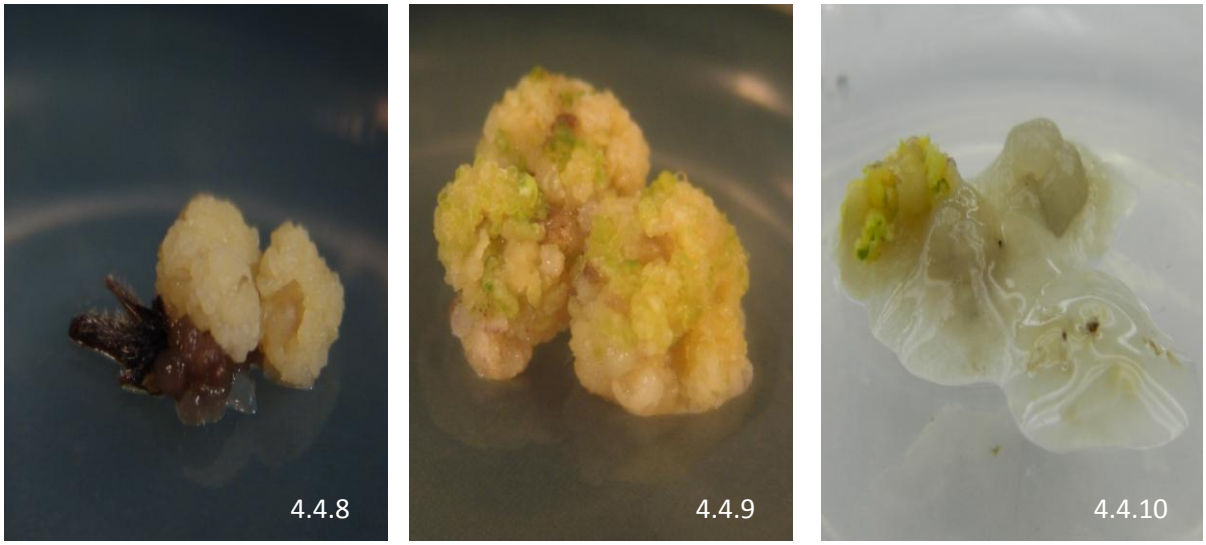



Figure 4.4.8 Induction of callus from nodal explants on MS medium supplemented with 2,4-D (29.22 µM) and coconut milk (10%); 4.4.9- Formation of nodular callus after 2 months; 4.4.10- Formation of mucilaginous callus

**4.4.4.1 Proliferation of callus**

After induction on higher concentration of 2,4-D (29.22 µM) and coconut milk (10%), callus was shifted to lower concentration of 2,4-D for proliferation. Callus proliferated at substantial rate on 14.61 µM 2,4-D without coconut milk. Incorporation of coconut milk was found to be essential for induction of callus only. Continuous exposure of callus to higher concentration of 2,4-D led to the formation of mucilaginous callus. On 2,4-D (14.61 µM), 12.53 g callus was obtained from initial lump of 3 g after 30 day of inoculation showing 4.16 fold increase in fresh weight. On lower concentration of 2,4-D (4.87 µM), only 5.2 g of callus was obtained after 30 days. Amount of callus lumps inoculated initially also had a direct effect on the callus growth rate. When 1 g of callus was transferred to fresh medium, only 1.5 fold increase was observed, whereas with 3 g of callus it increased to 4.16 folds. Effect of different concentrations of 2,4-D on callus proliferation are shown in table 4.4.3.

Table 4.4.3- Effect of different concentrations of 2,4-D on callus proliferation

| S.No | Media combinations | Fresh weight of callus after 30 days (g) | Multiplication fold |
|---|---|---|---|
| 1 | MS + 2,4-D (4.87 µM) | 5.20 ± 0.05 | 1.73 |
| 2 | MS + 2,4-D (9.74 µM) | 8.53 ± 0.08 | 2.84 |
| 3 | MS + 2,4-D (14.61 µM) | 12.53 ± 0.08 | 4.17 |
| 4 | MS +2,4-D (19.48 µM) | 9.80 ± 0.11 | 3.26 |
| 5 | MS + 2,4-D (24.35 µM)* | – | – |

*Rejected due to the formation of mucilaginous callus

**4.4.4.2 Organogenesis from callus**

When callus was shifted to lower concentration of 2,4-D (4.87 µM) in conjunction with BAP (1.13 µM), 40% callus lumps showed development of green protuberances but these did not grow into plantlets despite continuous culturing on the same medium. Histology of callus revealed the formation of meristemoids which developed into shoot bud initials (Figures 4.4.14, 4.4.15). But these initials failed to develop into shoots on any of the media tried. When such callus lumps were shifted to medium containing slightly higher concentration of BAP (2.26 µM), these lumps turned brown. Treatment with Kn, Zeatin or TDZ also proved ineffective as no shoot formation was observed. Addition of NAA (3.67 µM) to the 2,4-D (4.87 µM) supplemented medium could induce rooting only in the callus tissue. But continuous subculturing on the same medium resulted in browning of callus. Pulse treatment with BAP (2.26-44.3 µM), Kn (2.32 µM-23.25 µM), TDZ (0.45-22.5 µM) and Zeatin (0.91-9.12 µM) was also tried but regeneration of shoots and embryoid formation could not be achieved in the callus tissue.

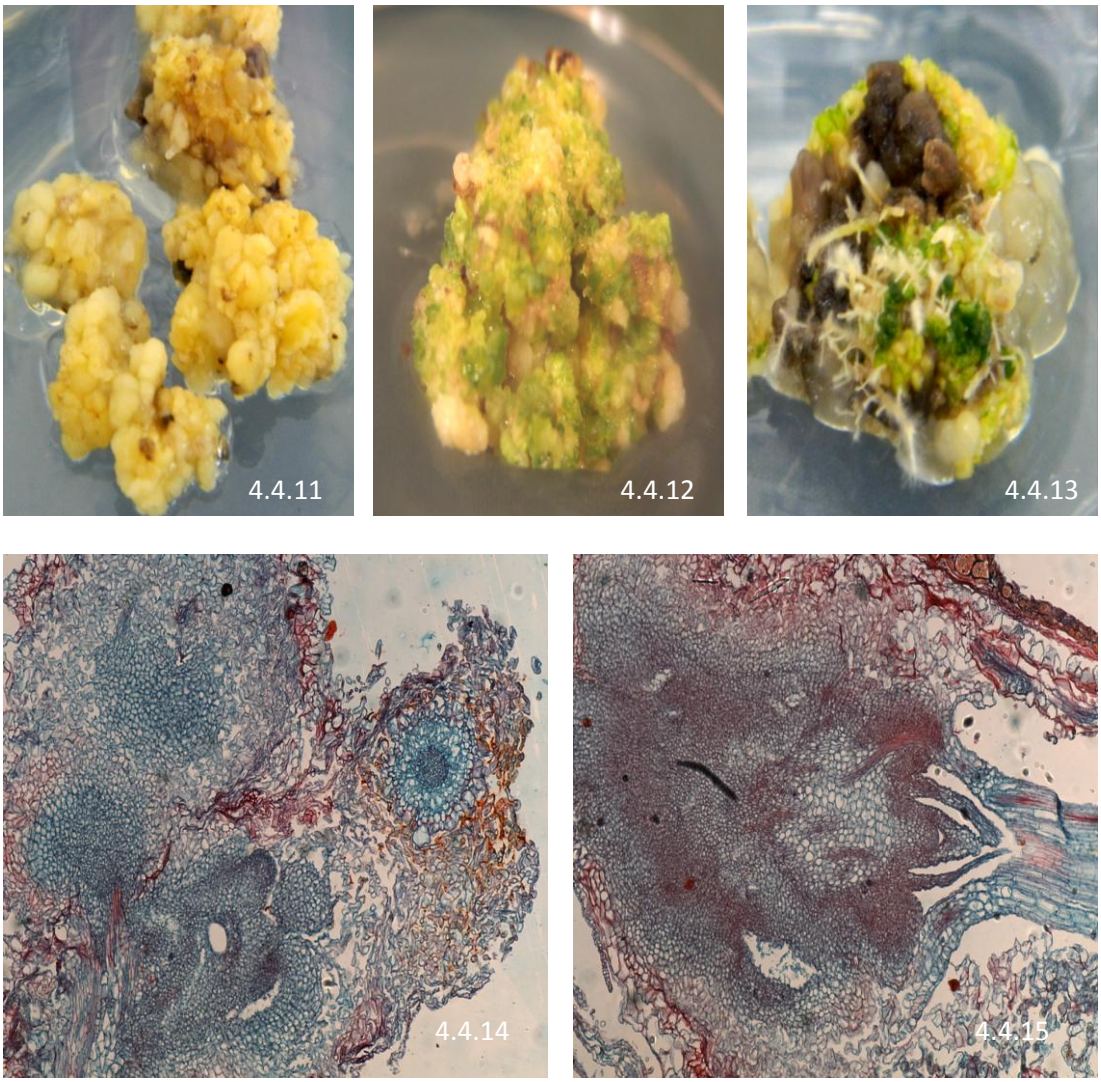



Figure 4.4.11- Callus proliferation on 2,4-D (14.61 µM); 4.4.12- Formation of green protuberances on 2,4-D (4.87 µM) + BAP (1.13 µM); 4.4.13- Formation of roots from callus on 2,4-D (4.87 µM) + NAA (3.67 µM); 4.4.14- Section of callus showing meristemoid formation; 4.4.15- Formation of shoot initials with subtending leaf primordia.

## Objective 2- To check the clonal fidelity of tissue culture raised plants by RAPD and to study their field performance

### 4.5 Clonal fidelity

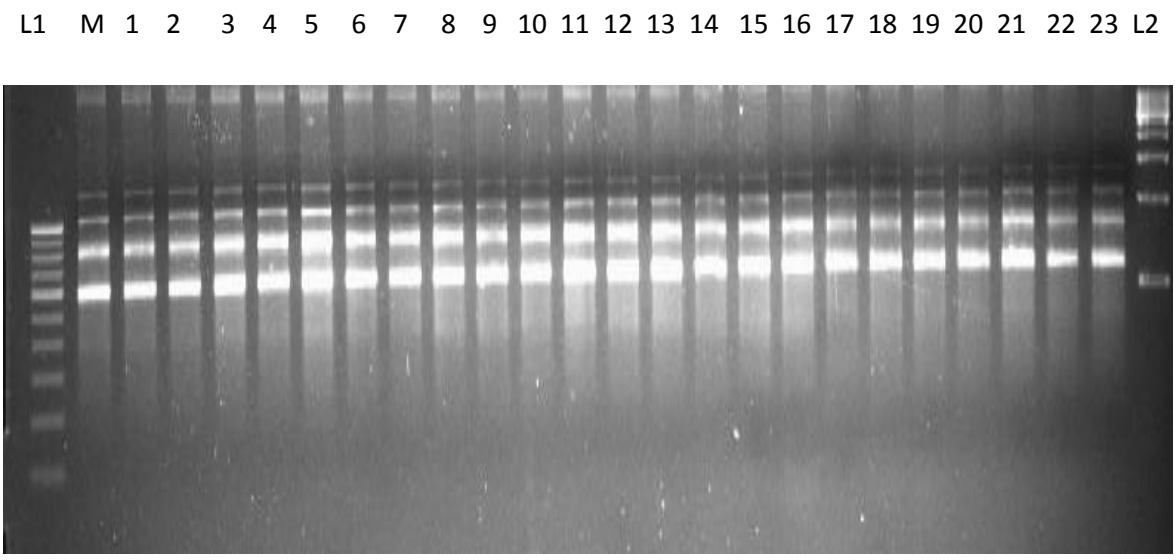
#### 4.5.1 Testing the clonal fidelity of *in vitro* raised *Dendrocalamus asper* plants

DNA samples from *in vitro*-grown shoots under various stages of subculture, hardened plants growing in the greenhouse, plants growing in the field and the mother plant were subjected to RAPD and ISSR analysis. Out of 27 ISSR primers used in initial screening, only 14 primers were found to be informative as they produced clear and reproducible bands. The 14 ISSR primers produced 40 distinct and scorable bands in the size range of 250 bp to 2400 bp (Table 4.5.1). The banding profiles from micropropagated plants were found to be monomorphic and similar to those of mother plant hence confirming their true to type nature (Figures 4.5.1 a,b). Likewise, during RAPD analysis out of 40 primers screened 30 primers produced clear and reproducible bands. These 30 RAPD primers produced 93 distinct and scorable bands in the size range of 200 bp to 2000 bp with an average 3.1 bands per primer (Table 4.5.2). No polymorphism was observed during RAPD analysis (Figures 4.5.2 a,b). These results confirmed that *D. asper* plants obtained by axillary branching method under *in vitro* conditions retained their clonal fidelity.

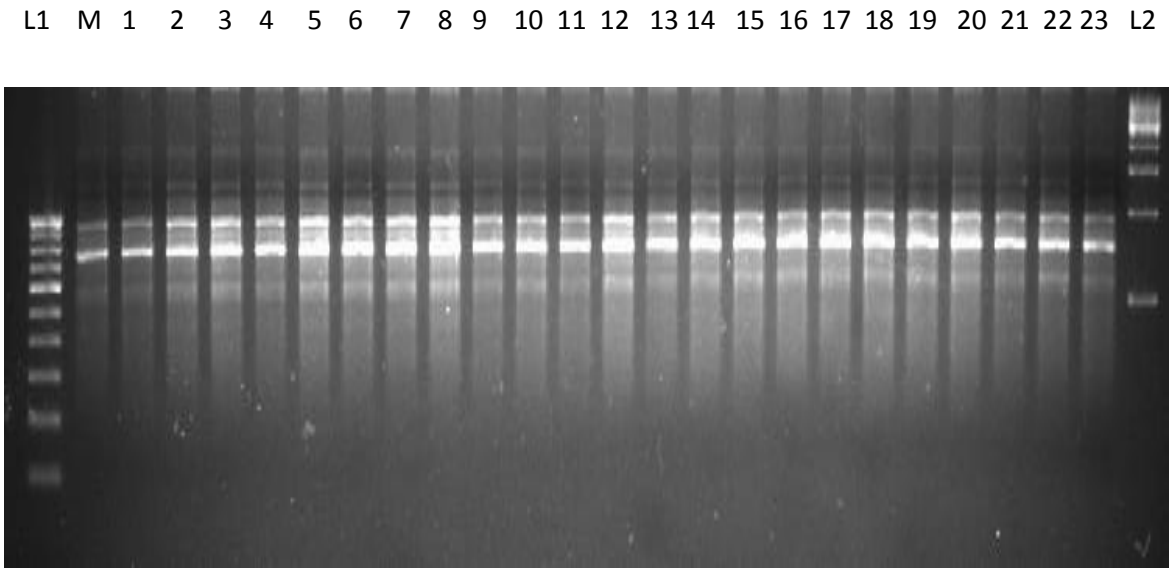
Table 4.5.1- The inter-simple sequence repeat (ISSR) primers utilized to verify *D.asper* clones.

| Primers | 5'-3' motif | Annealing Temperature($^{o}$C) | No. of scorable bands | Range of amplification (bp) |
|---|---|---|---|---|
| UBC 807 | AGAGAGAGAGAGAGAGT | 37.5 | 5 | 250-700 |
| UBC 811 | GAGAGAGAGAGAGAGAC | 38.3 | 4 | 1200-2400 |
| UBC 812 | GAGAGAGAGAGAGAGAA | 40.7 | 3 | 500-1100 |
| UBC 815 | CTCTCTCTCTCTCTCTG | 41.8 | 4 | 600-1400 |
| UBC 818 | CACACACACACACACAG | 47.1 | 1 | 1700 |
| UBC 836 | AGAGAGAGAGAGAGAGYA | 48.5 | 1 | 600 |
| UBC 841 | GAGAGAGAGAGAGAGAYC | 41 | 1 | 600 |
| UBC 842 | GAGAGAGAGAGAGAGAYG | 43.8 | 2 | 400-500 |
| UBC 843 | CTCTCTCTCTCTCTCTRA | 42.1 | 2 | 400-650 |
| UBC 848 | CACACACACACACACARG | 50.5 | 3 | 550-2100 |
| UBC 873 | GACAGACAGACAGACA | 42.4 | 5 | 600-2000 |
| UBC 888 | BDB CAC ACA CAC ACACA | 47.3 | 4 | 900-1800 |
| UBC 889 | DBDACACACACACACAC | 42 | 1 | 2000 |
| UBC 891 | HVHTGTGTGTGTGTGTG | 46.8 | 4 | 250-600 |

R=A+G; H=A+G; Y=C+T; V=G+A+C; B=G+T+C; D=G+A+T




(a)

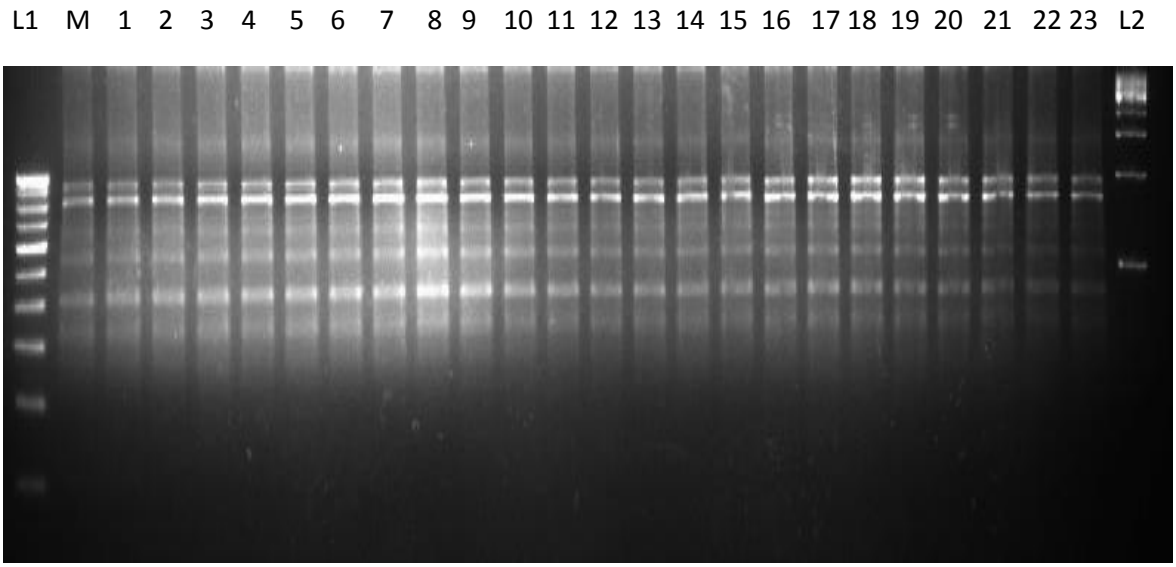



(b)

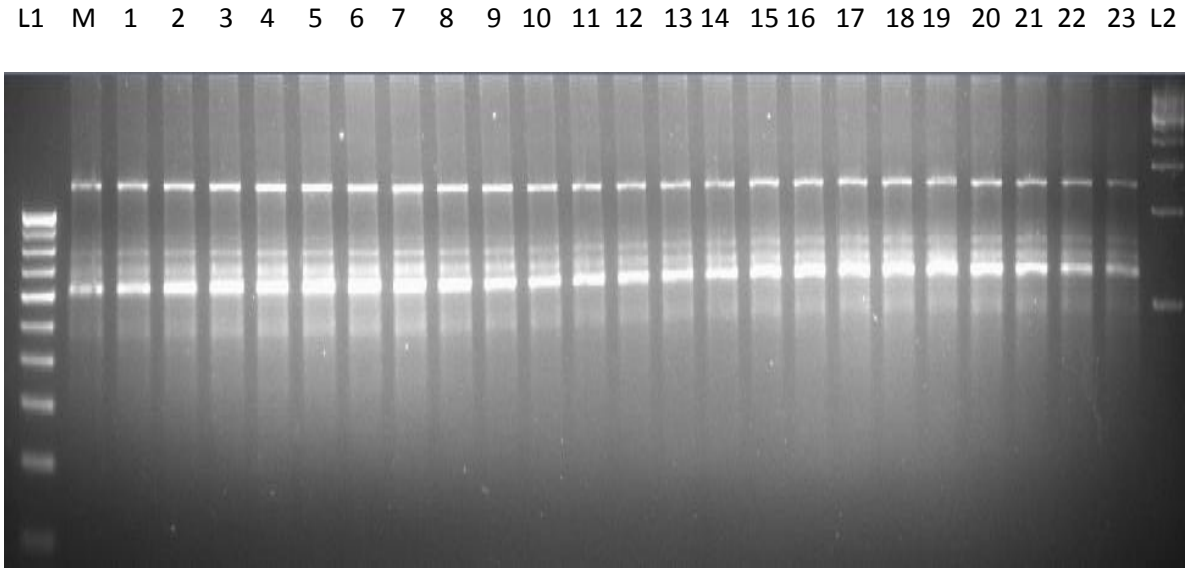
Figure 4.5.1- ISSR products generated from 23 *in vitro* regenerated plants and mother plants of *D.asper* amplified with primers (a) UBC 815 (b) UBC 873 showing monomorphic pattern. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1-23 represent *in vitro* raised clones of *D .asper* and Lane L2 represents 500-bp ladder.

Table 4.5.2- The random amplified polymorphic DNA (RAPD) primers utilized to verify *D. asper* clones.

| S.No. | Primers | 5'-3' motif | No. of scorable bands | Range of amplification (bp) |
|---|---|---|---|---|
| 1 | OPA 03 | AGTCAGCCAC | 1 | 700 |
| 2 | OPA 04 | AATCGGGCTG | 2 | 550-650 |
| 3 | OPA05 | AGGGGTCTTG | 2 | 700-900 |
| 4 | OPA 07 | GAAACGGGTG | 4 | 450-2000 |
| 5 | OPA 08 | GTGACGTAGG | 4 | 400-1300 |
| 6 | OPA 09 | GGGTAACGCC | 2 | 300-800 |
| 7 | OPA 10 | GTGATCGCAG | 2 | 450-550 |
| 8 | OPA 12 | TCGGCGATAG | 1 | 600 |
| 9 | OPC 06 | GAACGGACTC | 5 | 600-1500 |
| 10 | OPC 09 | CTCACCGTCC | 2 | 1200-1300 |
| 11 | OPC 10 | TGTCTGGGTG | 3 | 600-1200 |
| 12 | OPC 12 | TGTCATCCCC | 2 | 650-800 |
| 13 | OPC 13 | AAGCCTCGTC | 3 | 800-1500 |
| 14 | OPC 14 | TGCGTGCTTG | 6 | 350-900 |
| 15 | OPC 15 | GACGGATCAG | 7 | 300-1500 |
| 16 | OPC 16 | CACACTCCAG | 3 | 500-1200 |
| 17 | OPC 17 | TTCCCCCCAG | 2 | 200-500 |
| 18 | OPC 18 | TGACTGGGTG | 5 | 550-1400 |
| 19 | OPC 19 | GTTGCCAGCC | 3 | 600-1000 |
| 20 | OPC 20 | ACTTCGCCAC | 5 | 500-1300 |
| 21 | OPS 01 | CTACTGCGCT | 3 | 450-1500 |
| 22 | OPS 02 | CCTCTGACTG | 1 | 650 |
| 23 | OPS 03 | CAGAGGTCCC | 5 | 400-1200 |
| 24 | OPS 04 | CACCCCCTTG | 4 | 500-950 |
| 25 | OPS 05 | TTTGGGGCCT | 2 | 700-1400 |
| 26 | OPS 08 | TTCAGGGTGG | 2 | 600-900 |
| 27 | OPS 09 | TCCTGGTCCC | 1 | 450 |
| 28 | OPS 11 | AGTCGGGTGG | 3 | 300-850 |
| 29 | OPS 12 | CTGGGTGAGT | 6 | 200-1200 |




(a)




(b)

Figure 4.5.2 -Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA (RAPD) primer (a)OPC 14 (b) OPC 20. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1-23 represent *in vitro* raised clones of *D.asper* and Lane L2 represents 500-bp ladder.

### 4.5.2 Testing the clonal fidelity of *in vitro* raised *G. angustifolia* plants

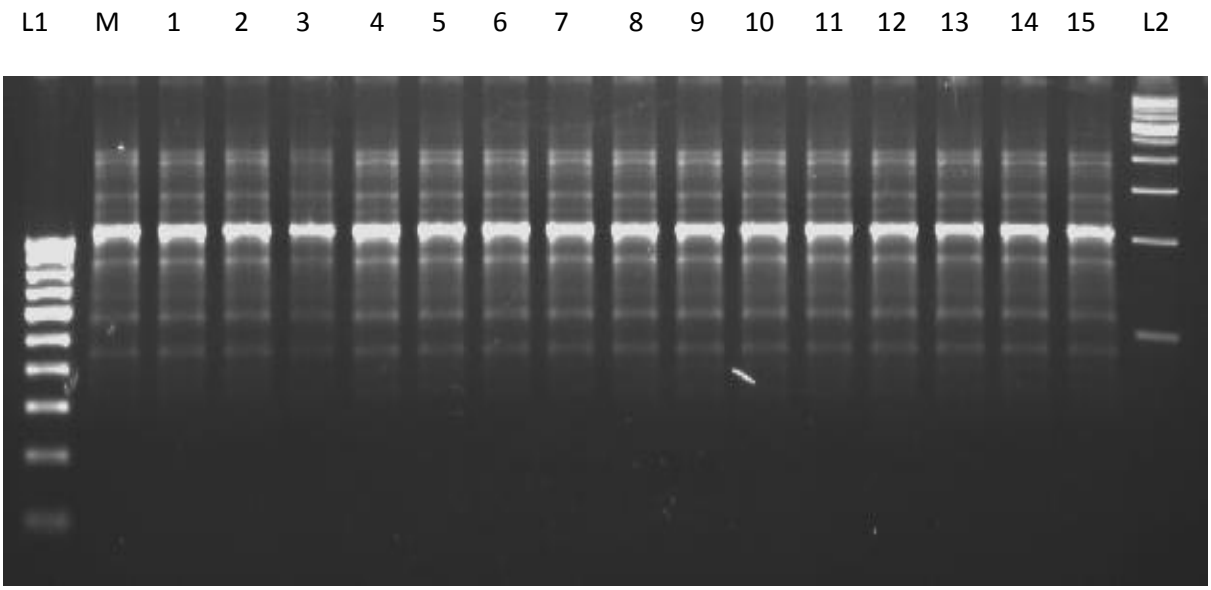
30 RAPD primers were used for initial screening with mother plant of *Guadua.* However, only 15 primers gave clear and reproducible bands. The number of

scorable bands for each primer varied from 2 (OPO-14) to 11 (OPT-17) (Table 4.6.3). The 15 RAPD primers produced 84 distinct and scorable bands in the size range of 200 bp to 2500 bp, with an average of 5.6 bands per primer. No polymorphism was observed during the RAPD analysis of *in vitro* raised clones (Figures 4.6.3 a,b).

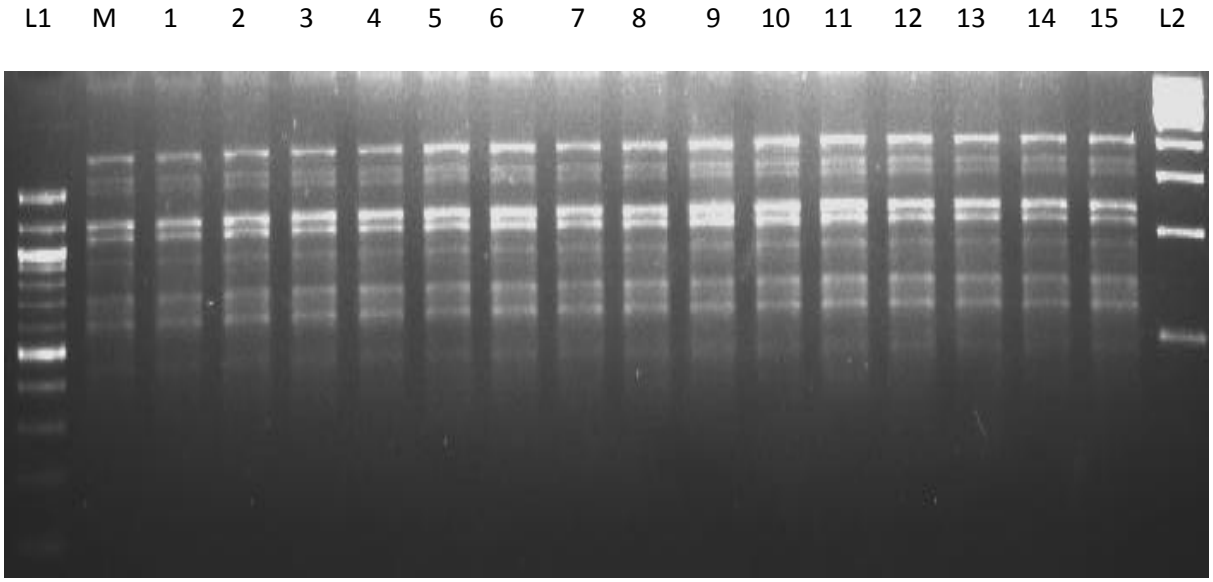
Out of 27 ISSR primers used in initial screening, only 17 primers produced clear and reproducible bands. The 17 ISSR primers produced 61 distinct and scorable bands in the size range of 300 bp to 2500 bp. The number of scorable bands of each primer varied from 1 to 7 (Table 4.6.4). The banding profiles from micropropagated plants were monomorphic and similar to those of mother plant (Figures 4.6.4 a,b).

Table 4.6.3- The random amplified polymorphic DNA (RAPD) primers utilized to verify *Guadua* clones.

| Serial no. | Primers | 5'-3' motif | No. of scorable bands | Range of amplification(bp) |
|---|---|---|---|---|
| 1 | OPO 02 | ACGTAGCGTC | 3 | 600-1200 |
| 2 | OPO 03 | CTGTTGCTAC | 8 | 400-2000 |
| 3 | OPO 04 | AAGTCCGCTC | 6 | 650-1700 |
| 4 | OPO 05 | CCCAGTCACT | 4 | 450-900 |
| 5 | OPO 06 | CCACGGGAAG | 5 | 550-2000 |
| 6 | OPO 10 | TCAGAGCGCC | 8 | 600-2000 |
| 7 | OPO 14 | AGCATGGCTC | 2 | 900-1100 |
| 8 | OPO 18 | CTCGCTATCC | 4 | 400-1300 |
| 9 | OPO 19 | GGTGCACGTT | 5 | 550-1600 |
| 10 | OPT 01 | GGGCCACTCA | 4 | 900-1800 |
| 11 | OPT 03 | TCCACTCCTG | 2 | 1500-2000 |
| 12 | OPT 07 | GGCAGGCTGT | 9 | 400-2100 |
| 13 | OPT 13 | AGGACTGCCA | 7 | 450-2100 |
| 14 | OPT 16 | GGTGAACGCT | 6 | 700-2500 |
| 15 | OPT 17 | CCAACGTCGT | 11 | 200-2000 |




(a)



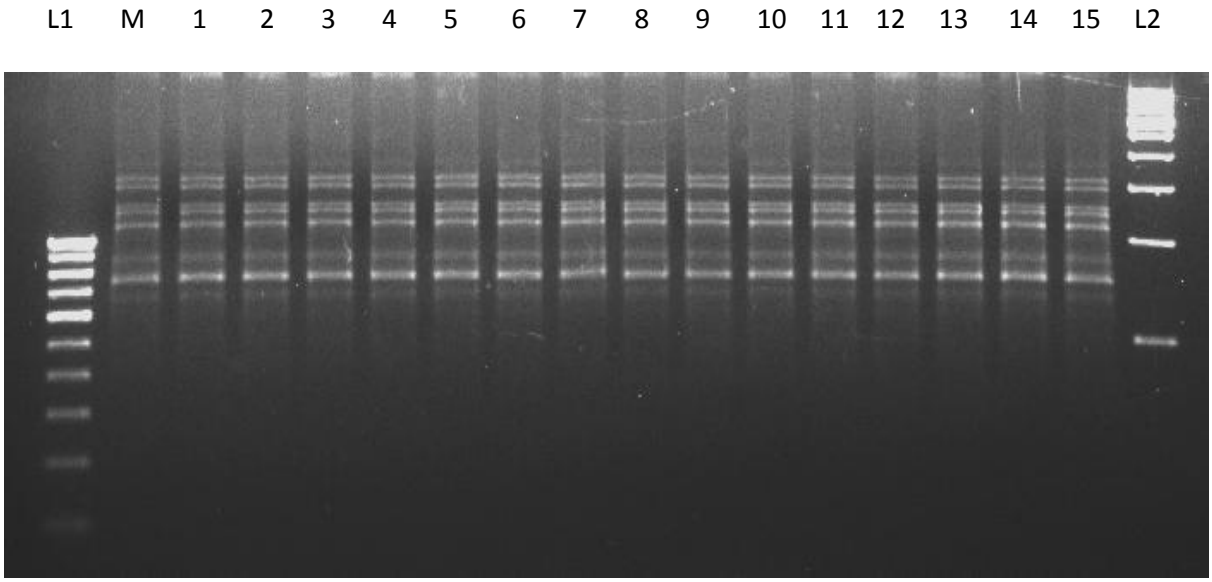

(b)

Figure 4.6.3 Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA (RAPD) primer (a) OPT-13 (b) OPO-10. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1-15 represent *in vitro* raised clones of *Guadua angustifolia* and Lane L2 represents 500-bp ladder.

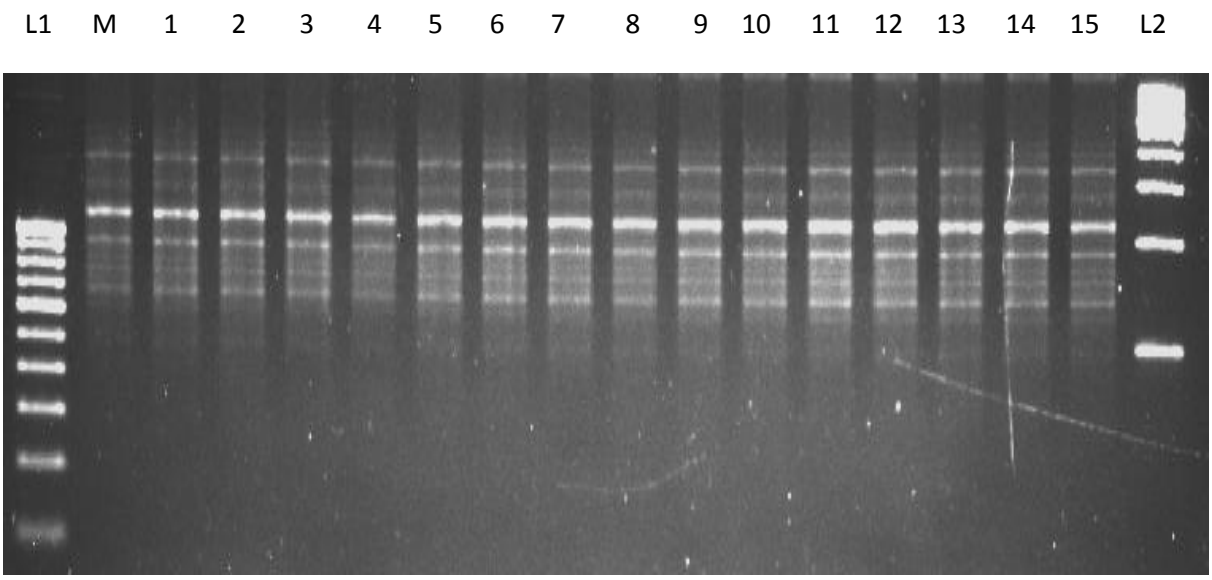
Table 4.6.4- The inter-simple sequence repeat (ISSR) primers utilized to verify *Guadua* clones.

| Serial no. | Primers | 5'-3' motif | Annealing Temperature ($^{o}$C) | No. of scorable bands | Range of amplification (bp) |
|---|---|---|---|---|---|
| 1 | UBC 807 | AGAGAGAGAGAGAGAGT | 37.5 | 6 | 300-700 |
| 2 | UBC 808 | AGAGAGAGAGAGAGAGC | 41.8 | 7 | 750-1600 |
| 3 | UBC 810 | GAGAGAGAGAGAGAGAT | 37.9 | 5 | 700-2500 |
| 4 | UBC 811 | GAGAGAGAGAGAGAGAC | 38.3 | 4 | 750-2000 |
| 5 | UBC 812 | GAGAGAGAGAGAGAGAA | 39.3 | 4 | 700-2000 |
| 6 | UBC 815 | CTCTCTCTCTCTCTCTG | 39.9 | 1 | 1500 |
| 7 | UBC 818 | CACACACACACACACAG | 47.1 | 3 | 1400-2500 |
| 8 | UBC 830 | TGTGTGTGTGTGTGTGG | 51.1 | 2 | 800-1500 |
| 9 | UBC 834 | AGAGAGAGAGAGAGAGYT | 40.4 | 2 | 800-1100 |
| 10 | UBC 835 | AGAGAGAGAGAGAGAGC | 41.8 | 4 | 850-1500 |
| 11 | UBC 840 | GAGAGAGAGAGAGAGAYT | 40.8 | 3 | 1100-1500 |
| 12 | UBC 841 | GAGAGAGAGAGAGAGAYC | 41 | 6 | 700-1700 |
| 13 | UBC 844 | CTCTCTCTCTCTCTCTRC | 41.5 | 1 | 1400 |
| 14 | UBC 848 | CACACACACACACACARG | 50.5 | 2 | 900-1450 |
| 15 | UBC 850 | GTGTGTGTGTGTGTGTYC | 48 | 1 | 1100 |
| 16 | UBC 873 | GACAGACAGACAGACA | 40 | 6 | 700-2500 |
| 17 | UBC 888 | BDBCACACACACACACA | 47.3 | 4 | 1000-1600 |

R=A+G; H=A+G; Y=C+T; V=G+A+C; B=G+T+C; D=G+A+T




(a)




(b)

Figure 4.6.4- ISSR products generated from 15 *in vitro* regenerated plants and mother plants of *G. angustifolia* amplified with primer (a) UBC 808 (b) UBC 841 showing monomorphic pattern. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1-15 represent in vitro raised clones of *G. angustifolia* and Lane L2 represents 500-bp ladder.

### 4.6 Analysis of field performance of *in vitro* raised plants

#### 4.6.1 Growth performance under green house conditions

During the first six months under green house conditions, maximum growth in terms of number of new shoots formed and height achieved was observed in *G.angustifolia* where on an average 9.32 new shoots emerged and maximum height achieved was 25.66 cm. In *D.asper* and *P. pubescens*, 6.32 and 4.66 new shoots emerged during this period respectively. The percentage survival of the plantlets under green house condition was 100% in *G. angustifolia,* 95% in *D. asper* and 80% in *P. pubescens.*

Table 4.6.1 Performance of the plants in the green house before transfer to the field

| **Species** | **Total number of shoots formed** | | | **Height of the longest shoot (cm)** | | |
|---|---|---|---|---|---|---|
| | After 2 months | After 4 months | After 6 months | After 2 months | After 4 months | After 6 months |
| *D .asper* | 2.33±0.33 | 1.66±0.33 | 2.33±0.33 | 17.66±0.88 | 21.33±0.88 | 25.66±0.88 |
| *G. angustifolia* | 2.66±0.33 | 4.33±0.33 | 2.33±0.57 | 16.66±0.88 | 22.00±1.15 | 27.33±1.20 |
| *P. pubescens* | 1.33±0.33 | 1.00±0.00 | 2.33±0.33 | 16.33±0.88 | 18.66±1.20 | 20.33±1.33 |

#### 4.6.2 Growth performance under field conditions

After successful growth and survival of the *in vitro* raised plants under green house conditions, plant were transplanted in the field to pits (2ft×2ft×2ft) at a plant to plant and row to row distance of 6 meters. Under field conditions, *D .asper* showed maximum growth attaining maximum height of 38.66 cm after 6 months and on an average 7.32 new shoots emerged during this period and registered 100% survival. In *P. pubescens,* 80% of the plants survived while in *G. angustifolia,* least survival rate of 30% was observed as *Guadua* being tropical bamboo could not bear

temperate climate of Palampur region. Hence, under field conditions *D. asper* showed maximum growth performance and survival rate followed by *P. pubescens.*

Table 4.6.2 Performance of the plants in the field

| **Species** | **Total number of shoots formed** | | | **Height of the longest shoot** | | |
|---|---|---|---|---|---|---|
| | After 2 months | After 4 months | After 6 months | After 2 months | After 4 months | After 6 months |
| *D .asper* | 3.00±0.00 | 1.66±0.33 | 2.66±0.33 | 28.00±1.00 | 33.00±1.52 | 38.66±1.33 |
| *G. angustifolia* | 1.00±0.00 | 1.00±0.57 | 0.00±0.00 | 29.66±1.45 | 31.00±1.52 | 32.66±1.45 |
| *P. pubescens* | 1.66±0.33 | 1.33±0.33 | 2.00±0.00 | 24.00±1.00 | 27.33±0.88 | 32.33±0.88 |

# Chapter 5
# Discussion

# 5 Discussion

Bamboos being tall, perennial, arborescent, fast-growing grasses have more than 1500 documented applications. Besides serving the rural communities through multiple uses, these are extensively used as raw material by paper industry. The immense utility and versatility of this natural resource has resulted in its over-exploitation leading to their scarcity. Erratic flowering, poor seed set, low seed viability, death of the whole plant/clump after gregarious flowering, indiscriminate extraction have posed a serious threat to its natural regeneration cycle. Due to multifarious uses, bamboos are attracting well deserved attention and their demand is increasing day by day. The wonderful feature of bamboo is that its culms are replenished after harvesting and generally very little or no post plantation care is required. Hence, they have been selected as priority species in India and many South-Asian countries.

In view of its growing demand and difficulties associated with traditional propagation methods, tissue culture offers rapid and reliable method for large scale production of selected plants. Under the scope of present investigation, micropropagation protocols for various bamboo species were standardized and different strategies for micropropagation were applied as per requirement and availability of propagules.

## 5.1 Micropropagation

**5.1.1 Culture initiation:** For the initiation of cultures, judicious selection of the explant is fundamental because different tissue types within the same plant differ in their response to a particular culture condition and the season of the year. In *Dendrocalamus asper*, among the various explants tested, unexpanded buds from tertiary branches were found to be the best for raising the cultures as they

responded favourably to different media combinations besides being easier to handle. In *Guadua angustifolia,* nodal segments from secondary and tertiary branches were used while in *Bambusa tulda*, nodal segments from secondary branches were used for raising cultures. In *Phyllostachys pubescens,* nodal segments obtained from mature plants failed to multiply on culture media, therefore, nodal segments from *in vitro* raised seedlings were used to initiate aseptic cultures.

Apical buds of secondary and tertiary branches could not sustain growth on the culture medium and turned necrotic. Young buds excised from secondary and tertiary branches planted on culture medium remained alive for one month but failed to sustain growth thereafter. Likewise, leaf explants did not show response on any of the media combinations tried and these became achlorophyllous in due course of time and perished.

Till date, 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, slow growth, poor multiplication rates and low rooting frequency etc. However, multiplication of superior bamboo clumps with desirable traits is possible only with the use of adult tissues than with seed/seedling explants. However, success with explants taken from mature plants of bamboos for propagation has been limited to a few cases only. Nadgir et al. (1984) successfully multiplied shoots derived from nodal explants of adult bamboos of the species *Bambusa bambos, B. vulgaris* and *Dendrocalamus strictus*. However, rooting occurred only in *D. strictus* with 20% efficiency. Later, Banik (1987) reported axillary shoot proliferation on medium supplemented with BAP (5µM), NAA (5.37 µM) and

activated charcoal (3g/l) but could not sustain further multiplication of these shoots. Subsequently, many other researchers viz. Chaturvedi et al. (1993), Saxena and Bhojwani (1993), Sood et al. (1992), Hirimburegama and Gamage (1995), Arya et al. (1998), Ramanayake and Yakandawala (1997), Ravikumar et al. (1998), Lin and Chang (1998), Mukunthakumar et al.(1999), Bag et al.(2000), Sanjaya et al. (2005), Ramanayake et al. (2006), Jimenez et al. (2006), Mudoi et al. (2009), Agnihotri et al. (2009) have also used nodal buds successfully for raising multiple shoots.

Multiplication from adult culms is restricted by many factors (Lin and Chang, 1998) and maturation of the tree species adversely affects the morphogenetic potential of the axillary buds (Pierik, 1990). Paranjothy et al. (1990) have highlighted some of the probable factors responsible for poor success with adult explants. These include microbial contamination, browning of the medium and inconsistency in shoot multiplication process.

**5.1.2 Browning of the medium**

According to Huang et al. (2002) tissue browning that frequently results in the early death of bamboo shoots *in vitro* is correlated directly with polyphenol oxidase activity. In their study involving *Bambusa oldhami* and *Phyllostachys nigra,* browning was clearly pH dependent and was influenced by initial pH of nutrient medium. Acidic nutrient medium with standard pH 5.7 gave relatively low browning rates. Das and Pal (2005) observed that the morphogenetic competence 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. 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. They reported that the morphogenetic competence of *B. balcooa* was moderately high from August to October, when total phenol content was comparatively low.

To avoid browning, activated charcoal and/or polyvinylpyrrolidone (PVP) are often added to the tissue culture media. Saxena and Dhavan (1999) reported that addition of PVP to the medium was effective in overcoming the browning of cultures in *D. strictus*. Ogita (2005) demonstrated that when the brown necrotic portions of tissues were removed from the explants and frequent subculturings were performed, browning was reduced considerably in *Phyllostachys nigra*. Similarly, Devi and Sharma (2009) observed occurrence of phenolic exudation at the cut ends of explants of *Arundinaria callosa* and recommended quick transfer to the fresh medium. Bisht et al. (2010) also recommended regular subculturing to fresh media at 2-3 days interval to overcome browning. In the present study, browning problem was overcome by frequent subculturing to the fresh medium.

**5.1.3 Microbial contamination**

Contamination in tissue culture is well documented (Leifert et al.1991) and the failure of surface sterilization procedures to produce aseptic cultures is a major problem with woody plants (Reed et al. 1998) and bamboos are no exception. The growth medium selected for *in vitro* propagation also serves as a good source of nutrients for microbial growth. These microbes further compete adversely with plants for nutrients (Odutayo 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, reduced shoot proliferation and reduced rooting (Kane, 2003). Ramanayake and Yakandawala (1997) observed an inverse relationship between *in vitro* bud break and systemic culture contaminants in *D. giganteus*. In *D. strictus* too,

culture initiation of mature field grown culms was difficult due to contamination. Our attempts to obtain aseptic *in vitro* cultures using explant of *Guadua* from glasshouse-grown plants were hindered by persistent appearance of bacterial contamination in the cultures. The contaminants were evident at the culture establishment stage and it resulted in the loss of plants when bacteria overgrew the explants. Incorporation of Kanamycin into the medium has been proved to be effective in controlling the growth of bacteria. Ramanayake and Yakandawala (1997) recommended the incorporation of Banlate in the medium to significantly reduce culture contaminants in *D. giganteus*.

Contamination rate has been found to be directly influenced by the amount of rainfall at the time of explants collection. In our study involving *D. asper* and *B. tulda,* highest rate of contamination was observed during the period of maximum rainfall (June-August). Hence, rainfall had a direct influence on contamination rates and survival percentage of explants.

**5.1.4 Bud break**

McClure (1966) observed that in nature, dormancy and breaking of dormancy in buds of bamboos varied with their position on the plant, the season of the year and the species. In *D. giganteus*, the mid-culm nodes of secondary branches have been reported to be the best explants for axillary shoot initiation (Ramanayake and Yakandawala, 1997). In *B. tulda*, single node cuttings from second and third internode of secondary branches were observed to be the best explants. In *G. angustifolia*, explants taken from higher branches were found to respond better on multiplication medium while in case of *D .asper*, mid culm nodes of tertiary branches proved better.

In *Bambusa vulgaris*, bud-break was induced more frequently throughout the year and was strong and positively correlated with rainfall (Kumari and Ramanayake, 1996). Saxena and Bhojwani (1993) found that *in vitro* bud-break in *Dendrocalamus longispathus* took place during the monsoon. 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. In tropical species including bamboos, changes in the environment such as those caused by rainfall or its onset may trigger the synthesis or breakdown of endogenous substances that control growth responses. But in our study involving *D. asper*, best period for initiating aseptic cultures was January and February when maximum bud break was achieved. In *B. tulda* explants collected during March-April gave better response in terms of increased bud break and early shoot proliferation, whereas in *G. angustifolia*, no such effect of season was observed as nodal explants collected at 15 day's intervals throughout the year, sprouted easily.

### 5.1.5 Cytokinins and bud break

According to Hirimburegama and Gamage (1995), cytokinins play an important role in inducing bud break. This study has been supported by many researchers later on. Among different cytokinins, BAP has been very effective in inducing sprouting of axillary buds in several bamboo species belonging to different genera (Prutpongse and Gavinlertvatana, 1992; Sood et al. 1992; Ramanayake and Yakandawala, 1997; Ravikumar et al. 1998; Bag et al. 2000). Godbole et al. (2002) demonstrated that incorporation of BAP in the medium could induce sprouting in nodal explants of *D. hamiltonii* within seven days. In *G. angustifolia*, in the absence of BAP, only 13% of the explants sprouted while highest sprouting rates (37.5%) were obtained with the addition of BAP (Jimenez et al. 2006). Presently, in *D .asper*, nodal explants cultured

on MS basal medium without any cytokinin took more time to sprout (25 days) and that too with low efficiency (30%). On the other hand, the nodal explants sprouted within 15 days of inoculation on MS medium supplemented with BAP (8.86 µM) in 90% of the cultures. We observed that the sprouting response declined with increase as well as decrease in the concentration of BAP. Similarly in *B .tulda* and *G. angustifolia,* incorporation of BAP was essential for inducing bud break.

**5.1.6 Seeds/Seedling explants**

After the pioneer report of establishing cultures of *D. strictus* using seeds (Alexander and Rao, 1968), 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).

Many successful reports employing seeds/seedling explants for raising cultures may be attributed to the easy disinfection of seeds. Paranjothy et al. (1990) listed various merits of using seed/seedling explants like low microbial contamination, possibility of year around initiation of cultures, relatively ease in achieving multiple shoots, high rooting ability and rare browning of shoots. 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. 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. 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. Moreover, the major

drawback with seedling explants is their inability to retain clonal uniformity in the plants thus produced. Moreover, in the absence of selection, the performance of a seed raised plant in the field with respect to growth and yield cannot be predicted in advance.

Due to our failure to induce nodal bud proliferation from mature plant under *in vitro* conditions, cultures were raised from nodal explants taken from *in vitro* raised seedlings in *P. pubescens*. Fungal contamination was quite rampant in *P. pubescens* seeds. The survival percentage of *in vitro* raised seedlings was 27.77%, but when the seeds were soaked in Bavistin (1%) and kept on a shaker overnight, their survival percentage improved to 43.05%. 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%) when seeds were used as explants but contamination rates declined when embryos were excised from the seeds and cultured separately.

**5.2 Shoot proliferation**

In *D. asper*, during preliminary experiments on MS basal medium, it led to necrosis and death of shoots in first passage. This necessitated the inclusion of cytokinins. On MS medium containing BAP (8.86 µM) in conjunction with adenine sulfate (13.5 µM), 48.66 shoots were formed after 60 days. Initially rate of multiplication was slow but it gradually increased during subsequent subcultures. The effectiveness of cytokinins especially BAP in promoting axillary shoot proliferation in forest trees as well as bamboos is well documented (Godbole et al. 2002; Arya et al. 2006; Kalia et al. 2007; Devi and Sharma, 2009; Bisht et al. 2010). 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).

In our study with *D. asper*, use of higher concentrations of BAP not only lowered the multiplication rate but also resulted in stunted shoots. Addition of kinetin alone did not result in any shoot proliferation. Instead, shoots remained dormant for some time and ultimately died. Negi and Saxena (2011) also reported the similar findings that higher concentration of BAP inhibited the shoot proliferation and addition of Kn did not promote shoot proliferation. Godbole (2003) reported that at higher concentration of BAP, the leaves remained curled up and failed to expand. Arya and Sharma (1998) had similar observation that higher doses of BAP induced thin leafy shoots. Vongvijitra (1988) reported inhibition of rooting with use of higher concentration of BAP during shoot formation. In some studies, higher concentrations of BAP have been found to induce negative effect on shoot length (Shirin and Arya, 2003; Shirin and Rana, 2007). Prutpongse and Gavinlertvatana (1992) highlighted that shoot lengths of *D. asper* genotypes decreased as BAP concentration was increased from 13.2 to 22 µM. Yashodha et al. (1997) also noticed the formation of stunted shoots in *B. nutans* and *D. membranaceus* with higher doses of BAP.

A high level of cytokinins is known to induce programmed cell death in cell cultures,yellowing of leaves and reduced root mass in intact plant (Carimi et al. 2003). Moreover, cytokinins often promote ethylene biosynthesis (Abeles et al. 1992) thus adversely affecting growth of tissue.

In our study involving *B. tulda*, when only BAP (13.29 µM) was used as growth promoter, 6.66 shoots were obtained after 60 days. But addition of Kn (4.65 µM) to BAP (8.86 µM) supplemented medium considerably enhanced the multiplication rate where 21.66 shoots were obtained after 60 days showing their synergistic effect.

Although initial growth rate remained slow upto 60 days, but increased with subsequent subcultures. Use of cytokinin BAP either alone or in combination with Kn or NAA in the basal medium has resulted in higher rate of shoot multiplication in several species of bamboos (Arya et al. 1998; Bag et al. 2000; Bag 2001; Sood et al. 2002b; Arshad et al. 2005; Kapoor and Rao, 2006; Ramanayake et al. 2006; Jimenez et al. 2006, Mudoi et al. 2009). A synergistic effect of BAP and Kn resulting in enhanced rate of multiplication has been observed in many species like *D. strictus*, *B. arundinacea* and *B. vulgaris* (Nadgir et al. 1984), *B. tulda* (Saxena, 1990; Das and Pal, 2005), *B. vulgaris* (Shirin et al. 2003), *D. strictus* (Ravikumar et al. 1998), *D. giganteus* (Ramanayaka and Yakandawala, 1997).

In *G .angustifolia*, MS liquid medium supplemented with BAP (8.86 µM) and adenine sulfate (13.5 µM) produced maximum lateral shoots with an average shoot number of 18.2 and shoot length of 7.06 cm after 90 days of culture. Addition of adenine sulfate to the culture medium can stimulate cell growth and greatly enhance shoot proliferation as it has a base structure similar to that of the cytokinins and hence, shows cytokinin-like activity.

In *P. pubescens,* shoot multiplication could not be achieved in sprouted buds of nodal explants, so the cultures were initiated using nodal segments of *in vitro* raised seedlings. Pulse treatment of shoots in TDZ supplemented medium was found to be beneficial. On an average 8 shoots were obtained when new sprouts were exposed to TDZ (0.9 µM) and Kn (4.65 µM) supplemented medium for 15 days followed by their transfer to MS basal medium. Increase in concentration of TDZ or exposure time to TDZ inhibited shoot formation. TDZ proved to be an effective cytokinin for proliferation in long term cultures of *B. oldhamii* (Lin et al. 2007a). In case of *Bambusa edulis*, TDZ was found to be effective for multiple shoot proliferation from

nodal explant (Lin et al. 1998) but its higher concentration inhibited elongation of shoots and led to considerable vitrification and albino shoot production. The presence of albino shoots in the tissue culture of grasses including bamboos is not uncommon (Vasil et al. 1990, 1993; Sood et al. 2002b). Stunting of shoots due to repeated culturing in TDZ supplemented medium has also been reported in *D. strictus* (Singh et al. 2000), *B. edulis* (Ramanayake et al. 2006) and *D. giganteus* (Ramanayake et al. 2001). Kim et al. (2004) reported that the regeneration of stunted and dwarf shoots in *Pyrus pyrifolia* is due to the expression of mRNA of dwarf gene in TDZ-treated leaves.

### 5.2.1 Propagules

During *in vitro* studies, we observed that the type of propagule (number of shoots per clump) used for shoot multiplication is a critical factor. Shoot clumps rather than single shoots were found to be effective for multiplication of bamboo plants (Arya et al.1999; Ramanayake et al. 2001, Sood et al. 1992). Bag et al. (2000) highlighted that propagules containing a minimum of three to four shoots proliferated at a maximum rate whereas single shoot would proliferate at a much slower rate. During micropropagation studies in *D. strictus*, Ravikumar et al. (1998) recommended that for further sustained growth and multiplication, the shoots should be transferred in groups of 5-7 during subculturing. In *D.asper*, we observed that six shoots per propagule were found most effective for multiplication where 27.2 shoots were obtained after 4 weeks of culture. Similarly, in *B .tulda,* 6 shoots per propagule were observed to be most favourable for shoot proliferation. Single shoots when used as propagule remained green for 2 weeks but failed to survive thereafter. Arya et al. (1999) reported that multiplication rate declined sharply if propagules of less than 3 shoots were cultured. Multiplication rate also declined if propagule of more than 3

shoots were used. But in our study involving *B. tulda* and *D. asper,* propagules with 6 shoots gave better performance.

### 5.2.2 Liquid medium

In *B. tulda* and *G. angustifolia,* multiple shoots were formed in liquid medium. Addition of gelling agent to multiplication medium reduced the multiplication rate in *B. tulda*. Further, shoots failed to survive due to necrosis after 30 days of culture. We found that cultures raised in liquid medium performed better than those raised on solid medium following the addition of a gelling agent (agar, gelrite, phytagel). According to Debergh (1983), solid medium causes binding of water and adsorption of minerals and growth hormones by gelling agents, thus restricting their supply to the growing shoots. On the other hand, liquid medium allows close contact with the tissues, which stimulates and facilitates the uptake of nutrients and phytohormones, leading to better shoot growth. Moreover, it is economical. Saxena (1990) also obtained the highest shoot multiplication rates and growth in liquid medium in *B. tulda.* In addition, shoots were greener and healthier in liquid medium than in semi-solid medium; lamina of leaf, which was almost inconspicuous or under developed on agar medium, was green and well developed in the liquid medium.

Several workers have reported higher rates of shoot multiplication and improved growth in liquid medium (Nadgauda et al. 1990; Sood et al. 1992; Ramanayake and Yakandawala, 1997; Ravi Kumar et al. 1998; Singh et al. 2001;Das and Pal, 2005; Sanjaya et al. 2005; Jimenez et al. 2006; Mishra et al. 2007; Negi and Saxena, 2011). Nadgir (1984) multiplied shoots in liquid medium and maintained the cultures on a shaker for entire shoot multiplication cycle of 6-7 weeks. In *D. giganteus*, liquid cultures in shaker responded faster to axillary shoot proliferation than stationary cultures (Ramanayake and Yakandawala, 1997). But in the present study, the

necessity of shaker to provide aeration to shoots was avoided by reducing amount of liquid medium in the culture vessel and frequent subculturing at 15 days interval. However, a continuous culture in liquid medium may cause vitrification of shoots as reported by Saxena and Bhojwani (1993) in *D. longispathus*. Ramanayake and Yakandwala (1997) used luffa pieces to support shoots while Saxena and Bhojwani (1993) controlled vitrification of shoots by supporting them on pieces of foam. Negi and Saxena (2011) avoided the problem of hyperhydration by supporting shoot clusters using shredded Whatman filter paper strips. However, no vitrification was observed in our cultures when quantity of liquid was kept below the level of first node sufficient enough to submerge the base of shoots. But prolonged culturing in the liquid medium resulted in necrosis of the leaves and leaf sheath due to submerged conditions.

### 5.2.3 Gelling agent

During propagation of *D. asper* and *P. pubescens*, replacement of the solidified medium with liquid medium during multiple shoot proliferation did not prove effective. Use of phytagel instead of agar was found to be beneficial for promoting shoot multiplication. The use of agar lowered multiplication rate and reduced the length of shoots as compared to phytagel solidified medium. Bag et al. (2000) pointed out that culturing in agar gelled medium resulted in secretion of phenolics by explants and was associated with browning of medium and reduced multiplication. They recommended the use of medium gelled with phytagel which resulted in comparable multiplication efficiency. Similarly, Ramanayake and Yakandwala (1997), Zamora et al. (1988), Agnihotri et al. (2009) recommended the use of phytagel for healthy growth of plants.

### 5.2.4 Carbon source

Effect of carbon source (Sucrose, Table sugar and Glucose) on shoot multiplication was also studied. In *D. asper,* although sucrose (3%) was found to be most suitable carbon source as 48.2 shoots were obtained with 3.22 cm shoot length after 6 weeks of culture but 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. In *P. pubescens,* glucose was preferred carbon source as 8 shoots were obtained after 4 weeks of culture. Use of 3% sucrose has been recommended for shoot proliferation in many bamboo species viz *D. giganteus* (Ramanayake and Yakandawala, 1997), *D. hamiltonii* (Sood et al. 1992), *D. asper* (Arya et al. 1999), *G. angustifolia* (Jimenez et al. 2006), *B .oldhamii* (Lin et al. 2007a), *B. tulda* (Mishra et al. 2007), *B. vulgaris* (Ndiaye et al. 2006), *B. glaucescens* (Shirin and Rana, 2007).

In *B. tulda,* 2% sucrose proved to be ideal for shoot multiplication both in terms of number as well as their overall growth. At 1% sucrose, the number of new shoots declined and shoots did not look healthy. Increased levels of sucrose (3%) did not affect shoot number but shoots turned necrotic and ultimately died. On glucose fortified medium, least multiplication was achieved and shoots failed to survive after second subculture cycle. Addition of 2% sucrose has been advocated by many authors viz. Nadgir et al. 1984 (*D. strictus*, *B. arundinacea* and *B. vulgaris*), Saxena 1990 (*B. tulda*), Shirgurkar et al. 1996 (*D. strictus*); Ravikumar et al. 1998 (*D. strictus*); Agnohotri et al. 2009 (*D. hamiltonii*). Saxena (1990) reported that incorporation of higher levels of sucrose during micropropagation of *B. tulda* caused severe albinism, while in *B. bambos* increase in sucrose concentration resulted in more number of rhizome formation (Kapoor and Rao, 2006)

### 5.2.5 Coconut milk

Many researchers have reported the promotory effect of coconut milk on bamboo shoot cultures and emphasised incorporation of coconut milk into the multiplication medium (Nadgir et al. 1984). Saxena and Bhojwani (1993) observed that use of coconut milk (10%) enhanced the shoot multiplication in *D. longispathus.* Nadgauda et al. (1990) induced shoot proliferation in seedling derived cultures of *B. arundinacea* and *D. brandisii* in medium supplemented with coconut milk. Ramanayake and Yakandawala (1997) observed that in the absence of coconut milk, a significantly lower number of shoots were obtained in *D. giganteus.* Coconut milk is reported to have cytokinins and their effect on shoot proliferation may have been due to the availability of nutrients and growth regulators (George et al. 1984). In our study incorporation of coconut milk into shoot multiplication medium did not enhance proliferation rate significantly.

## 5.3 Rooting

For the development of any successful micropropagation protocol, root induction from excised shoots and subsequent survival of plantlets in the soil are crucial. Rooting is a major bottleneck while carrying out *in vitro* studies in bamboos. It was observed that rooting was more effectively induced when cluster of shoots rather than individual shoot were used. In *D. asper*, on lower concentration of IBA (2.46 µM) only 10% rooting was observed while on IBA (14.76 µM) maximum rooting response (50%) was discerned. With further increase in concentration of IBA development of roots declined and propagules failed to survive. Addition of NAA to the IBA supplemented medium proved effective as maximum rooting (90%) was achieved with an average of 5.66 roots per propagule and mean root length of 3.06 cm after 45 days of culture.

Auxins when used in combination are known to enhance rooting frequency significantly. Many reports are available in which different combinations and permutations of auxins were employed to induce rooting in bamboos (Negi and Saxena, 2011; Islam and Rahman, 2005; Arya et al. 2006).

Even a number of authors have recommended the incorporation of coumarin to auxin supplemented media to enhance rooting. Saxena and Bhojwani (1993) had successfully used coumarin for rooting of microshoots of *D. longispathus* in combinations with IBA and NAA. In *B. tulda*, Saxena (1990) reported root induction in shoots raised from seeds in the presence of coumarin. But in our study, incorporation of coumarin was not required as 90% rooting was achieved on combination of IBA and NAA in *D. asper*. Nadgir et al. (1984) and Sood et al. (2002a) reported that the incorporation of activated charcoal alone in the medium also induced rooting. But in our case no rooting was observed on medium supplemented with charcoal. Instead shoot turned necrotic and died.

In *G. angustifolia*, auxins were not required for inducing rooting as root formation was achieved simultaneously with shoot multiplication on multiplication medium in 100% of cultures. A continuous and exponential development of new roots was observed after 45 days of culture initiation. On the other hand, Jimenez et al. (2006) had reported rooting of *Guadua* plantlets spontaneously after 69 days in culture. In *D. strictus,* Shirgurkar et al. (1996) also induced rooting from shoots on medium supplemented with BAP without any addition of auxins. Similarly, Vongvijitra (1988) reported rooting on basal medium or in the presence of low concentration of BAP while carrying out micropropagation studies in *D. brandisii*.

In *P. pubescens*, a two step procedure was applied for root induction. Initial exposure to IBA (9.84 µM) for 15 days and subsequent withdrawal of auxin from the

medium resulted in root induction in almost 75% of cultures within 4 weeks of transfer to auxin free medium. Continuous culture on medium with higher doses of IBA for relatively longer periods adversely affected growth and subsequent survival of shoots. The same strategy was used by Bag et al. (2000) for inducing rooting in *Thamnocalamus spathiflorus.* In *D. hamiltonii*, rooting efficiency was markedly enhanced when propagules were placed on to IBA supplemented MS medium for 10 days and then transferred to IBA free medium (Agnihotri et al. 2009).

*B. tulda*, attempts were made to induce rooting by inoculating the clump of three to four shoots to various concentrations and combinations of auxins (IBA, IAA and NAA). But rooting was not achieved on any of these auxins. Even addition of coumarin (Mishra et al. 2007) into the medium failed to induce rooting. 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. Incorporation of activated charcoal also proved ineffective. In bamboos, differences in rooting performance may be attributed to their varied inherent genetic ability to root both *in vitro* and *ex vitro*.

**5.4 Acclimatization**

After the plantlets were established *in vitro,* they were subjected to hardening under green house conditions. These plantlets were transferred to pots containing river bed sand and covered with jars to maintain high humidity (80-85%) for 25-30 days before transferring to potting mixture. In *D. asper*, the plants were transferred into potting mixture containing sand:soil:manure in different ratios (v/v;1:1:1; 1:1:2; 1:0:0; 0:0:1 ). The plants transplanted in pots containing 1:1:1 (sand:soil:manure) potting mixture showed a successful hardening rate of 95%. The plants when shifted to field

conditions exhibited 100% survival. Arya et al. (1999) obtained 95% survival rate after transplantation of *D. asper* plants raised through seeds.

In *G. angustifolia*, 100% of plants survived during hardening phase. Hardened plants had green and healthy leaves with expanded lamina. Rao et al. (1990) reported that hundred percent survival could be achieved if plantlets developed rhizome. But, Sood et al. (1992) indicated that pre formation of rhizome was not mandatory for survival of rooted plants. In our study, 100% survival was attained without any formation of rhizome. Furthermore, we standardized the procedure by indicating that the plantlets bearing 4-5 shoots were more amenable to hardening than with less or more shoots.

In *P. pubescens,* four week old rooted plantlets were used for transplantation. Only 30% of the rooted plants directly transferred to the potting mixture (sand:soil:manure;1:1:1) survived while 80% survival was achieved when the plantlets were shifted to sand covered with jars where high humidity around plants was maintained before transferring them to the potting mixture. Saxena (1990) also reported less survival rate in plants when they were directly transferred to potting mixture. Their survival rate increased (80-90%) by including initial stage of controlled humidity around the plant.

**5.5 Clonal Fidelity**

Although the i*n vitro* germplasm conservation depends on micropropagation technique, the phenotypic and genetic variations may occur during *in vitro* propagation and subsequently may give rise to somaclonal variants (Kaeppler et al., 2000). Therefore, it is essential to assess the clonal stability of *in vitro* derived plants to obtain genetically pure elites rather than having indifferent populations (Eshraghi et al. 2005; Chandrika and Rai, 2009; Mehta et al. 2010). Many factors are

responsible for inducing variability during tissue culture such as explants source, time of culture, number of subcultures, phytoharmone, genotype, media composition, the level of ploidy and genetic mosaicism (Silvarolla, 1992). The activation of transposable elements (Hirochika et al. 1996), DNA hypomethylation (Jaligot et al. 2000; Keyte et al. 2000; Lukens and Zhan, 2007), 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.

Among various methods of *in vitro* propagation, the axillary shoot proliferation is least susceptible to genetic modification (Shenoy and Vasil, 1992). However, the possibility of somaclonal variations cannot be ruled out even with this method. Plant tissue culture is regarded as a major area of biotechnology because of its potentiality to regenerate elite genetic resources but scaling up of any micropropagation protocol is severely hindered due to incidences of somaclonal variations (Larkin and Scowcroft, 1981), methylation, chromosome rearrangements and point mutations (Phillips et al., 1994b). The occurrence of somaclonal variation is a potential drawback when propagation of an elite plant species is intended. Here the clonal fidelity is essential to maintain the advantages of desired elite genotype (Rahman and Rajora, 2001; Nadha et al. 2011).

There are very few reports which can confirm the clonal fidelity of bamboo plantlets derived from axillary bud proliferation. The scarcity of reports on ascertaining the genetic fidelity of tissue culture raised plantlets can jeopardise the quality of micropropogated plants, especially in perennials like bamboo 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.

Earlier, Das and Pal (2005) established the clonal fidelity of regenerants of *Bambusa tulda* and *B. balcooa* using only four markers to assess their genetic uniformity among the regenerants. Godbole (2003) assessed the clonal fidelity of *in vitro* raised plants of *D. hamiltonii* using 18 RAPD markers. Later, Negi and Saxena (2010) employed 15 ISSR markers to validate the clonal fidelity of *in vitro* raised *B. balcooa* plantlets through the axillary bud proliferation. However, there is no report available on the comparative genetic stability of regenerants and mother plant of *D. asper* and *G. angustifolia* by using RAPD and ISSR markers. In the present study, we did not find any polymorphism during the RAPD analysis of *in vitro* raised clones of *D. asper* and *G. angustifolia.* In *G. angustifolia,* 15 RAPD primers produced 84 distinct and scorable bands in the size range of 200 bp to 2500 bp, with an average of 5.6 bands per primer. While in *D. asper,* during RAPD analysis out of 40 primers screened 30 primers produced clear and reproducible bands. These 30 RAPD primers produced 93 distinct and scorable bands in the size range of 200 bp to 2000 bp with an average 3.1 bands per primer. This is consistent with the absence of genetic variations observed during micropropagation of *Pinus thunbergii* (Goto et al. 1998), turmeric (Salvi et al. 2001), Lilium (Varshney et al. 2001), *Bambusa balcooa* and *Bambusa tulda* (Das and Pal, 2005), *Dendrocalamus hamiltonii* (Agnihotri et al. 2009) and Gerbera (Bhatia et al. 2011), analyzed using RAPD markers.

In *G. angustifolia*, we screened 27 ISSR primers, however only 17 ISSR primers produced 61 distinct and scorable bands in the size range of 300 bp to 2500 bp. In addition, the banding profiles from micropropagated plants were monomorphic and similar to those of mother plant. Similarly in *D. asper* no polymorphic bands were obtained during ISSR analysis, thus confirming true to type nature of *in vitro* raised plants. Similar results have been reported in *Bambusa balcooa* (Negi and Saxena,

2010), *Bambusa nutans* (Mehta et al. 2010; Negi and Saxena, 2011), gerbera (Bhatia et al. 2011).

Mehta et al. (2010) assessed the genetic variations of field-established *B. nutans* plants raised through somatic embryogenesis by AFLP using 6 primer combinations and recorded a high level of genetic stability. Out of four hundred and seven scorable fragments amplified, 402 (98.8%) recorded conservation at various morphogenetic stages leading to plantlets regeneration.

### 5.6 Field performance of *in vitro* raised plants

The benefit of any micropropagated system can only be fully realized by the successful establishment of the plants to *ex vitro* conditions. A substantial number of micropropagated plants do not survive when transferred to field conditions as plants get exposed to stressful environment.

In the present investigation, *D. asper* showed 100% survival under field conditions and registered a maximum growth in terms of height achieved (38.66 cm) and number of new shoots emerged (7.32). In *P. pubescens,* 80% of the plants survived while in *G. angustifolia,* least survival rate of 30% was observed as *Guadua* being tropical bamboo could not bear temperate climate of Palampur region.

Performance of *in vitro* raised plants under field conditions had been highlighted in many reports. In *D. hamiltonii*, Agnihotri et al. (2009) attained 70% survival rate and observed sixfold increment in plant height in 18 months. In *B. nutans*, Negi and Saxena (2011) 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 *D.asper*, Arya et al. (1999) achieved 95% survival in plants raised through seeds.

## 5.7 Callus induction and proliferation

Induction of callus has been reported from different explants like leaves in *B. multiplex* (Jullien and Van, 1994), anthers in *Sinocalamus latiflorus* (Tsay et al. 1990), from roots of *B. beecheyana* Munro var . beecheyana (Chang and Lan, 1995), from nodal segment in *B. vulgaris* (Rout and Das, 1997; Gielis, 1999), *B. ventricosa* (Gielis, 1999), *D. hamiltonii* (Godbole et al. 2002), *B. nutans* (Mehta et al. 2010) and from inflorescences of *B. oldhamii* Munro (Yeh and Chang, 1986a), *B. beecheyana* Munro var. beecheyana (Yeh and Chang, 1986b), *B. edulis* (Lin et al. 2004a). In the present study involving *D. asper* and *B. tulda,* callusing could only be induced in explants taken from *in vitro* raised shoots only. Moreover, it was found that the age of *in vitro* raised shoots was critical for induction of callus. Only 30 days old shoots which developed by subculturing of shoot propagules were competent for callusing.

2,4-D was observed to be the most commonly used auxin for inducing embryogenic callus in bamboos (Mehta et al. 1982; Rao et al. 1985; Yeh and Chang, 1986a,b; Rout and Das, 1994; Godbole et al. 2002; Arya et al. 2009; Mehta et al. 2010). There are some reports depicting use of other auxins like picloram and NAA for inducing callus but such calli were friable and non-regenerant. Huang et al. (1988) observed that picloram was ten times more potent than 2,4-D for cell growth in suspension cultures of B. oldhamii but beyond the exponential growth period, picloram caused yield to decline very sharply. They further reported that in *B. multiplex*, picloram caused deadening of cells when used repeatedly during subculture. In the present investigation in *D. asper,* callus formation started at cut ends of the nodal explants after 15 days under dark conditions on MS medium supplemented with 2,4-D (14.61 µM) and within 8 weeks the whole explants turned into a mass of callus. The callus was capable of sustained growth on the same medium. In *B. tulda,* callus could only

be induced on the higher concentration of 2,4-D (29.22 µM) together with coconut milk (10%). Incorporation of NAA or BAP to the callus induction medium was found to be inhibitory. After induction, callus was shifted to lower concentration of 2,4-D for proliferation. Callus proliferated at substantial rate on 14.61 µM 2,4-D without coconut milk. Incorporation of coconut milk was found to be essential for induction of callus only. Continuous exposure of callus to higher concentrations of 2,4-D lead to the formation of mucilaginous callus. On 2,4-D (14.61 µM), 12.53 g callus was obtained from initial lump of 3g after 30 day of inoculation showing 4.16 fold increase in fresh weight.

In *B. tulda*, two type of calli were observed 1) nodular and compact 2) mucilaginous and transparent. Occurrence of such calli has also been reported by many workers (Rao et al. 1985; Rout and Das 1994; Wood et al. 1992; Yeh and Chang, 1987; Saxena and Bhojwani, 1993; Chang and Lan, 1995; Godbole et al. 2002 and Arya et al. 2009). However, in *D. asper* only nodular and compact calli were obtained.

### 5.7.1 Differentiation from callus

In *D. asper*, shoot differentiation was observed when callus was shifted to 2,4-D (9.74 µM) and BAP (1.13 µM) supplemented medium. Only in 20% of cultures, shoot differentiation was achieved. The callus was observed to be highly rhizogenic as numerous thick white roots differentiated from callus after 30 days covering the entire callus mass after 90 days. Histological studies revealed the formation of globular meristemoids which developed into shoot initials. In earlier reports, addition of cytokinins such as Kn, BAP proved beneficial in inducing somatic embryos in some bamboo species (Wood et al. 1994; Godbole et al. 2002; Arya et al. 2009; Zhang et al. 2010). Godbole et al. (2002) induced somatic embryogenesis in the callus of *D. hamiltonii* by gradually decreasing the concentration of 2,4-D in the

medium with corresponding increase in the concentration of BAP. But in the present study, treatment with cytokinins didnot prove effective as stages of embryoid formation could not be traced out. Even addition of auxins (IAA, IBA, NAA) either alone or in combination with cytokinins promoted only extensive rooting in the callus. Arya et al. (2009) could induce somatic embryogenesis in *D. asper* on combination of 2,4-D (9 µM), IAA (2.85 µM) and BAP (0.88 µM). However, in our study use of above combinations failed to induce somatic embryogenesis.

In *B. tulda,* development of green protuberances was observed when callus was shifted to combination of 2,4-D (9.74 µM) and BAP (2.26 µM) but these failed to grow into plantlets. Attempts were also made to induce somatic embryogenesis but it remained unsuccessful. It is opined that bamboo cells are undoubtedly totipotent but they require trigger for organ formation. We firmly believe that some vital hormonal and or nutritional factor essential for differentiation in bamboos is yet to be discovered.

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# Chapter 6
# Conclusion

# 6 Conclusion

The commercial importance of bamboos has attracted a number of investigators to devise methods for the rapid multiplication of clones with superior traits, since the conventional methods of seed and vegetative propagation by offsets and culm cuttings have proved inefficient for most of the bamboo species. Consequently, tissue culture is fast developing as a promising technique to obtain genetically pure elite populations under *in vitro* conditions. In contrast to herbaceous dicotylednous and monocotylednous angiosperms which have been extensively studied through tissue culture and morphogenetic techniques, bamboos have received rather scant attention. However, in recent years, the realization of the potential of tissue culture techniques in tree improvement program has considerably accelerated research in bamboos too.

The bamboos still pose challenges in the area of use of adult explants, efficient rooting of microshoots, enhanced conversion rates of somatic embryos, direct organogenesis, *in vitro* flowering, pollination and production of viable hybrid seeds and also to understand the mechanism of enigmatic bamboo flowering. Consequently, more tissue culture work is required to explore the physiological mechanism of rooting and growth processes. Although the technique of tissue culture remains to be commercially feasible for most of the bamboo species, the promise and potential of mass clonal propagation in near future cannot be underestimated. It is hoped that commercial production of bamboos would become a reality in not too distant a future.

# Chapter 7

# Summary

# 7 Summary

Bamboos, one of the natural resources of the tropics are fascinating plants because of their wide distribution, availability, rapid growth, easy handling and desirable properties. For billions of people in Asia, South-America and also Africa, bamboos are an integral part of their daily life requirements and their use from ‘cradle to the coffin’ is an often cited proverb. Bamboos are utilized intensively for a wide range of purposes. Because of this, today bamboos are much overexploited plants causing shortage of raw materials and this has forced greater attention to propagation practices and techniques. It is in this context that the present work had been initiated.

As the bamboos are cross pollinated, a lot of heterogeneity is observed in the seedling population which may be important for biodiversity conservation but highly unsuitable for economic planatation. Therefore, prior selection of field grown plants was ensured before attempting their mass propagation. The selection was based on the growth performance of plants under field conditions and the nodal segments from precocious branches of field grown plants proved to be the best explants for initiating aseptic cultures.

Breaking the dormancy of buds varied with their position on the plant and season of the year, as midculm nodes of tertiary branches were found to be the best explants for axillary shoot proliferation. Moreover, the rate of contamination in the cultures also varied with season. Rainfall had a direct influence on contamination rates and survival percentage of explants. For establishing aseptic cultures, seasonal constraints associated with budbreak and appropriate collection time of explants was also taken care of. In *Dendrocalamus asper*, best period for initiating aseptic cultures

was January and February when maximum bud break was achieved. Whereas in *B. tulda,* explants collected during March-April gave better response.

For large scale multiplication of *Bambusa tulda*, *Dendrocalamus asper*, *Guadua angustifolia*, *Phyllostachys pubescens* under *in vitro* conditions, nodal segments were collected from field grown elite mother plants of known age and subjected to surface sterilization using various sterilizing agents. The explants were thereafter inoculated onto MS medium supplemented with different cytokinins for inducing bud break. The sprouted buds were then excised from nodal segments and transferred onto MS medium containing different concentrations and combinations of cytokinins for multiple shoot proliferation

An efficient procedure for multiple shoot proliferation was achieved from axillary buds when cultured on cytokinins supplemented medium. Prolific shoot multiplication was achieved in *D. asper* where nearly 48.66 shoots were regenerated per axillary bud when cultured on MS medium supplemented with BAP (8.86 µM) and adenine sulphate (13.5 µM). In *B. tulda,* 21.66 shoots were obtained when BAP (8.86 µM) was used in conjunction with Kn (4.65 µM). In *G. angustifolia,* 18.2 shoots were formed when BAP (8.86 µM) and adenine sulphate (13.5 µM) were used synergistically. Bacterial contamination was the major problem encountered during initiation of aseptic cultures in *G. angustifolia*. Subculturing of the infected shoots onto the multiplication medium containing antibiotic (Kanamycin) for 10 days and thereafter shifting to the medium without any antibiotic, proved to be beneficial for eliminating bacterial contamination. In *P. pubescens,* bud break was observed only in the explants taken during December and January but multiple shoot proliferation could not be achieved on any concentration and combination of cytokinins or auxins tried. Hence, experiments were carried out using seeds. Nodal explants from *in vitro*

raised seedlings were used for initiating multiple shoots. Although multiple shoots were obtained but their rate of proliferation was slow as only 7-8 shoots could be obtained after eight weeks of culture.

For inducing callus, *in vitro* raised shoots were excised into small pieces (0.5-1cm) and transferred onto MS medium supplemented with different auxins and cytokinins. Incorporation of 2,4-D into the medium was found to be essential. Callus formation started at the cut ends of the explants within 15-20 days. The frequency of induction varied with concentration of 2,4-D in the medium. In *B. tulda*, callus was obtained on MS medium supplemented with 2,4-D (29.22 µM) and coconut milk (10%) . Moreover, calli formed were of two types- fast growing mucilaginous and comparatively slow growing nodular and compact. In *D. asper* only nodular and compact callus was achieved on 2,4-D (14.61 µM).

In *D. asper*, differentiation of shoots from callus was observed when the latter was shifted to MS medium supplemented with 2,4-D (9.74 µM) and BAP (1.13 µM). Histological studies were carried out which revealed the formation of meristemoids in the callus tissue. The callus tissue was found to be highly rhizogenic. In *B. tulda*, callus tissue showed the development of green protuberances but these did not grow into plantlets on any of combinations tried.

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. asper*, root formation was observed in 90% of cultures on medium containing IBA (14.76 µM) and NAA (3.67 µM). In *G. angustifolia*, rooting was achieved on the multiplication medium without any addition of auxin in 100% of the cultures. While in *P. pubescens*, initial treatment of shoots in IBA (9.84 µM) supplemented medium for 15 days followed by

their transfer to auxin free medium proved to be effective for inducing rooting. Continuous treatment of shoots with auxin for more than 15 days resulted in browning of the shoots. In *B. tulda*, rooting could not be achieved on any combinations of auxins as the shoots failed to survive. Even pulse treatment to the shoots in auxin supplemented medium did not prove to be beneficial for inducing rooting. Attempts were also made to induce rooting *ex vitro* but no rooting could be achieved.

To test the clonal fidelity, the *in vitro* raised plants at various stages of subculture and hardened plants growing in the field were chosen randomly. In *G. angustifolia,* 30 RAPD primers were used for initial screening. However, only 15 primers gave clear and reproducible bands. The number of scorable bands for each primer varied from 2 (OPO-02) to 11 (OPT-17). No polymorphism was observed during the RAPD analysis of *in vitro* raised clones. Out of 27 ISSR primers used in initial screening, only 17 primers produced clear and reproducible bands. The number of scorable bands for ISSR primer varied from 1 to 7. The banding profiles from micropropagated plants were monomorphic and similar to those of mother plant, thereby, confirming true to type nature of *in vitro* raised plants.

In *D. asper*, out of 40 RAPD primer screened initially, 29 primers were found to be informative as they produced clear and reproducible bands. While in case of ISSR primers, 14 primers were found to be informative out of 27 primers screened. No polymorphism was observed during RAPD and ISSR analysis of *in vitro* raised clones.

For analysing the performance of *in vitro* raised plants under field conditions, growth parameters like number of shoots, length of longest shoot of *in vitro* raised plants

were recorded after hardening stage. Their percentage of survival was calculated. Growth and multiplication data of such hardened plantlets was recorded for the first six months under the green house conditions. These plants were then transferred to the field and their performance with respect to number of culms and height of longest culm was recorded for another six months under field conditions.

During the first six months under green house conditions, maximum growth in terms of number of new shoots formed and height achieved was observed in *G.angustifolia* followed by *D. asper* and *P. pubescens.* The percentage survival of the plantlets under green house condition was 100% in *G. angustifolia,* 95% in *D. asper* and 80% in *P. pubescens.* Under field conditions, *D. asper* and *P. pubescens* registered the survival of 100% and 80% respectively. *G. angustifolia* being tropical bamboo could not bear the temperate climate of Palampur region and exhibited the least survival rate of 30%. Under field condition, *D .asper* showed maximum growth attaining maximum height of 38.66 cm after 6 months and on an average 7.32 new shoots emerged during this period.

Thus we have successfully established the protocols for micropropagation of *Dendrocalamus asper* and *Guadua angustifolia* from nodal explants derived from mature plants. Moreover, we have also ascertained the clonal fidelity of *in vitro* raised plants of *D. asper* and *G. angustifolia* through RAPD and ISSR markers. To our knowledge, we are the first one to report *in vitro* propagation of *P. pubescens* using nodal segments of *in vitro* raised seedlings. Thus our methods can be used for rapid and mass propagation of important bamboo species.

# Chapter 8
# References

# 8 References

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## Annexure I

## MURASHIGE AND SKOOG's (1962) MEDIUM

| Constituents | Concentrations(mg/l) |
|---|---|
| Macroelements | |
| $NH_4NO_3$ | 1650.0 |
| $KNO_3$ | 1900.0 |
| $CaCl_2 . 2H_2O$ | 440.0 |
| $MgSO_4 . 7H_2O$ | 370.0 |
| $KH_2PO_4$ | 170.0 |
| $FeSO_4$ | 27.85 |
| $Na_2EDTA$ | 37.25 |
| Microelements | |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $H_3BO_3$ | 6.20 |
| $CoCl_2.6H_2O$ | 0.025 |
| $CuSO_4.5H_2O$ | 0.025 |
| $ZnSO_4$ | 8.6 |
| $MnSO_4$ | 22.3 |
| KI | 0.83 |
| Vitamins | |
| Glycine | 2.0 |
| Mesoinositol | 100.0 |
| Nicotinic acid | 0.5 |
| Pyridoxine-HCl | 0.5 |
| Thiamine-HCl | 1.0 |

## Annexure II

Meteorological data of Palampur during 2008

| | Temp | Vapour pressure (mm) | | Relative humidity | | Rainfall | Evaporation |
|---|---|---|---|---|---|---|---|
| | Mean | VP (M) | VP (E) | RH (M) | RH (E) | mm | Mm |
| January | 8.85 | 5.1 | 7.8 | 71 | 62 | 136.5 | 2.2 |
| February | 10.5 | 4.9 | 6.8 | 67 | 56 | 98.5 | 2.6 |
| March | 18.6 | 7.7 | 5.4 | 46 | 26 | 15.4 | 4.9 |
| April | 19.95 | 7.8 | 7.3 | 47 | 31 | 59 | 5.5 |
| May | 23.3 | 10.9 | 10.3 | 51 | 37 | 180.1 | 6.4 |
| June | 22.9 | 16.3 | 18.1 | 81 | 73 | 526.1 | 3.1 |
| July | 22.6 | 17.5 | 19.5 | 86 | 82 | 538 | 2.4 |
| August | 21.55 | 17 | 18.8 | 87 | 84 | 607 | 2.3 |
| September | 21.0 | 14 | 17.7 | 84 | 73 | 89 | 3.2 |
| October | 19.15 | 10.4 | 13.4 | 73 | 57 | 65.4 | 3.2 |
| November | 15.4 | 6.3 | 8.4 | 60 | 44 | 0 | 2.7 |
| December | 14.05 | 5.5 | 7.4 | 58 | 46 | 9.2 | 2.4 |

Source- Himachal Pradesh Agriculture University, Palampur (H.P), India

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*Full Length Research Paper*

# Evaluation of clonal fidelity of *in vitro* raised plants of *Guadua angustifolia* Kunth using DNA-based markers

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***Guadua angustifolia*** **Kunth a large and multipurpose bamboo has extensive utility in pharmaceutical, paper, charcoal, and construction industries. Therefore, it is pertinent to scale up their production through micropropagation technique. This would enable us to meet the growing demand for quality planting material. Although, the common practice to use axillary bud method allows large-scale production, there are always possibilities of somaclonal variations which appear in *in vitro* cultures due to its rapid multiplication. Therefore, we utilized random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers in *in vitro* raised *Guadua* clones to assure their genetic fidelity. We screened 30 RAPD and 27 ISSR primers, and found 15 RAPD and 17 ISSR markers to produce clear, reproducible and scorable bands. We found 15 RAPD primers which produced 84 distinct bands with an average of 5.6 bands per primer. In addition, we found 17 ISSR primers which produced 61 distinct bands in the size range of 300 to 2500 bp. All 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.**

**Key words:** *In vitro* propagation, *Guadua* clones, DNA markers.

## INTRODUCTION

Bamboos, the giant grasses with woody culms growing uprightly, are widely grown plant source for medicinal products, food, fiber, shelter, and building materials. Traditionally, the bamboos have been used for treating infectious diseases and wound healing. The bamboo leaves are also used for counteracting spasmodic disorders. Inefficient and labour intensive conventional methods for propagating bamboos, gregarious flowering habit, poor seed set and low viability, and human population pressure disrupting the natural cycle of reforestation present an urgent need for developing efficient methods for large scale propagation of bamboos. Overexploitation of bamboo has led to the rapid depletion of natural strands hence, generating a grave concern about conservation as well as to develop propagation methodologies for new plantations and reestablishment of cleared strands. Limitations in traditional propagation methods, such as the use of offsets, branch cuttings together with unpredictable and long flowering cycle (about 35 years), warrants an urgent need for an alternative approach for developing efficient and reproducible protocols for its mass propagation. Given the difficulties of conventional propagation techniques, *in vitro* propagation provides a promising alternative.

Among the various methods of *in vitro* propagation, the axillary shoot proliferation is a least susceptible to genetic modification (Shenoy and Vasil, 1992). However, the possibility of somaclonal variations cannot be ruled out even with this method as reported in *Populus deltoids* (Rani et al., 1995), *Robinia pseudoacacia* (Bindiya and Kanwar, 2003), *Hagenia abyssinica* (Feyissa et al., 2007), *Olea europaea* (Peyvandi et al., 2009). Plant tissue culture is regarded as a major area of biotechnology because of its potentiality to regenerate elite genetic resources but scaling up of any

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micropropagation protocol is severely hindered due to incidences of somaclonal variations (Larkin and Scowcroft, 1981). Somaclonal variations are induced due to the stress imposed on the plant during propagation and is incorporated in the form of DNA methylation, chromosome rearrangements and point mutations (Phillips et al., 1994). The occurrence of somaclonal variation is a potential drawback when propagation of an elite plant species is intended. Here, the clonal fidelity is essential to maintain the advantages of desired elite genotype (Rahman and Rajora, 2001). Several techniques are available to assess tissue culture induced variations in plants such as morphological descriptions, physiological supervisions, cytological studies, isozymes (Gupta and Varshney, 1999) and molecular markers. However, the molecular markers are regarded as rapid, sensitive and more reliable alternative approach (Sharma et al., 2008).

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). RAPD and ISSR markers are simple, fast, cost effective, highly discriminative and reliable. ISSR circumvents the requirement for flanking sequence information and thus, has wide applicability in a variety of plants (Srivastava and Gupta, 2008). The ISSR markers have also been useful in establishing genetic stability of several micropropagated plants, such as cauliflower (Leroy et al*.,* 2000), *Populus tremuloides* (Rahman and Rajora, 2001), *Swertia chirayita* (Joshi and Dhawan, 2007) and *Dictyospermum ovalifolium* (Chandrika et al., 2008), *Ochreinauclea missionis* (Chandrika and Rai, 2009), *Bambusa balcooa* (Negi and Saxena, 2010). Similarly, RAPD markers have been applied for characterisation of micropropagated forest trees viz. *Picea mariana* (Isabel et al., 1993), *Populus deltoids* (Rani et al., 1995), Oak (Barrett et al., 1997), *Populus tremuloides* (Rahman and Rajora, 2001).

Here, we show a method to assess the clonal fidelity of *in vitro* raised *Guadua angustifolia* plants using RAPD and ISSR markers. This will provide us with a useful tool for establishing a unique micropropagation system for the production of genetically identical and stable plants before they are released for large scale plantations or other commercial purposes.

## MATERIALS AND METHODS

Nodal segments of 2 to 4 cm in length obtained from 4 year old potted plant were used for initiating aseptic cultures. Murashige and Skoog's (1962) (MS) medium supplemented with BAP (6-Benzylaminopurine) (2 mg/L) was used for inducing bud break. The *in vitro* derived shoots were cultured on MS medium supplemented with BAP (2 mg/L) and adenine sulphate (10 mg/L) for proliferation and multiplication. The rooting was also obtained on the same multiplication medium. The cultures were maintained at 25 ± 2°C under fluorescent white light (70 µmol/$m^2$/s) during a photoperiod of 16:8 h light and dark cycle. The rooted plantlets were hardened on sand and soil mixture (1:1) under greenhouse conditions.

### DNA extraction and PCR amplification

To test the clonal fidelity, the *in vitro* raised plants at various stages of subculture along with the hardened plants were chosen randomly. These plants were compared with the mother plant from which explants were taken. Total genomic DNA of the mother plant and *in vitro* raised plants was extracted from young leaf tissue by using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990). Thirty RAPD primers and twenty seven ISSR primers (Sigma-Aldirch, Bangalore, India) were used for initial screening. PCR amplifications were carried out in total volume of 25 µl containing 2 µl (20 to 25 ng) of genomic DNA. The reaction buffer for ISSR consisted of 2.5 µl Taq buffer, 1 µl $MgCl_2$, 0.15 µl dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP), 1.5 µl primer, 0.17 µl Taq polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 17.68 µl water. PCR amplification was performed in a DNA thermal cycler, which was programmed for initial DNA denaturation at 94°C for 4 min, followed by 44 cycles of 1 min denaturation at 94°C, 1 min annealing (temperature specific to the primer) and 1 min extension at 72°C, with a final extension at 72°C for 7 min. For RAPD, reaction buffer consisted of 2.5 µl Taq buffer, 0.5 µl $MgCl_2$, 0.2 µl dNTPs, 1.5 µl primer, 0.17 µl Taq polymerase and 18.13 µl water. PCR amplification consisted of initial denaturation at 94°C for 5 min, followed by 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 37°C and 2 min extension at 72°C, with a final extension at 72°C for 7 min. The amplified products were resolved by electrophoresis on 1.8% agarose gel in tris-borate EDTA (TBE) buffer stained with ethidium bromide. The fragment sizes were estimated with 100 and 500 bp DNA ladders (Bangalore Genei Pvt. Ltd, Bangalore, India)

## RESULTS

We used 30 RAPD primers for initial screening with mother plant of *Guadua.* However, only 15 primers generated clear and reproducible bands. The number of scorable bands for each primer varied from 2 (OPO-14) to 11 (OPT-17) (Table 1). The 15 RAPD primers produced 84 distinct and scorable bands in the size range of 200 to 2500 bp, with an average of 5.6 bands per primer. We did not observe any polymorphism during the RAPD analysis of *in vitro* raised clones (Figure 1). Out of 27 ISSR primers used in initial screening, only 17 primers produced clear and reproducible bands. These 17 ISSR primers produced 61 distinct and scorable bands in the size range of 300 to 2500 bp. The number of scorable bands for each primer varied from 1 to 7 (Table 2). We found that all banding profiles from micropropagated plants were monomorphic and similar to those of mother plant (Figure 2).

## DISCUSSION

Although, the *in vitro* germplasm conservation depends on

**Table 1.** The random amplified polymorphic DNA (RAPD) primers utilized to verify *Guadua* clones.

| S/N | Primers | 5'-3' motif | No. of scorable bands | Range of amplification (bp) |
|---|---|---|---|---|
| 1 | OPO 02 | ACGTAGCGTC | 3 | 600-1200 |
| 2 | OPO 03 | CTGTTGCTAC | 8 | 400-2000 |
| 3 | OPO 04 | AAGTCCGCTC | 6 | 650-1700 |
| 4 | OPO 05 | CCCAGTCACT | 4 | 450-900 |
| 5 | OPO 06 | CCACGGGAAG | 5 | 550-2000 |
| 6 | OPO 10 | TCAGAGCGCC | 8 | 600-2000 |
| 7 | OPO 14 | AGCATGGCTC | 2 | 900-1100 |
| 8 | OPO 18 | CTCGCTATCC | 4 | 400-1300 |
| 9 | OPO 19 | GGTGCACGTT | 5 | 550-1600 |
| 10 | OPT 01 | GGGCCACTCA | 4 | 900-1800 |
| 11 | OPT 03 | TCCACTCCTG | 2 | 1500-2000 |
| 12 | OPT 07 | GGCAGGCTGT | 9 | 400-2100 |
| 13 | OPT 13 | AGGACTGCCA | 7 | 450-2100 |
| 14 | OPT 16 | GGTGAACGCT | 6 | 700-2500 |
| 15 | OPT 17 | CCAACGTCGT | 11 | 200-2000 |

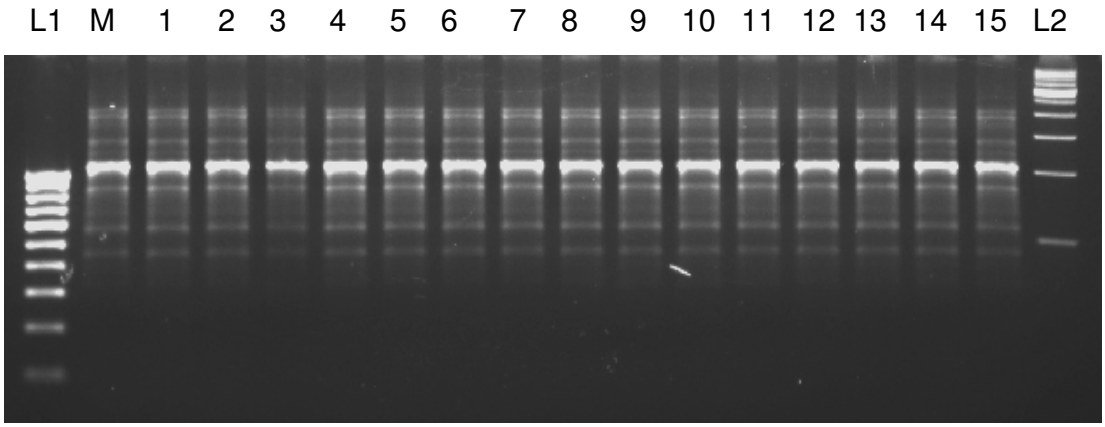


**Figure 1.** Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA (RAPD) primer (OPT-13). Lane L1 represents 100 bp ladder, Lane M represents mother plant, Lane 1 to 15 represent *in vitro* raised clones of *Guadua angustifolia* and Lane L2 represents 500 bp ladder.

micropropagation techniques, the phenotypic and genetic variations may occur during *in vitro* propagation and subsequently may give rise to somaclonal variants (Kaeppler et al., 2000). Therefore, it is essential to assess the genetic stability of *in vitro* derived clones for micropropagation of true-to-type clones (Eshraghi et al., 2005, Chandrika and Rai, 2009). Many factors are responsible for inducing variability during tissue culture, such as explants source, time of culture, number of subcultures, phytoharmone, genotype, media composition, the level of ploidy and genetic mosaicism (Silvarolla, 1992). The activation of transposable elements (Hirochika et al., 1996), DNA hypomethylation (Jaligot et al., 2000; Keyte et al., 2000; Lukens and Zhan, 2007), 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 the previous variations (Peyvandi et al., 2009). There are very few reports which can confirm the clonal fidelity of bamboo plantlets derived from axillary bud proliferation. The scarcity of reports on ascertaining the genetic fidelity of tissue culture raised plantlets can jeopardise the quality of micropropogated plants, especially in perennials like bamboo 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.

**Table 2.** The inter-simple sequence repeat (ISSR) primers utilized to verify *Guadua* clones.

| S/N | Primers | 5'-3' motif | Annealing Temperature (°C) | No. of scorable bands | Range of amplification (bp) |
|---|---|---|---|---|---|
| 1 | UBC 807 | AGAGAGAGAGAGAGAGT | 37.5 | 6 | 300-700 |
| 2 | UBC 808 | AGAGAGAGAGAGAGAGC | 41.8 | 7 | 750-1600 |
| 3 | UBC 810 | GAGAGAGAGAGAGAGAT | 37.9 | 5 | 700-2500 |
| 4 | UBC 811 | GAGAGAGAGAGAGAGAC | 38.3 | 4 | 750-2000 |
| 5 | UBC 812 | GAGAGAGAGAGAGAGAA | 39.3 | 4 | 700-2000 |
| 6 | UBC 815 | CTCTCTCTCTCTCTCTG | 39.9 | 1 | 1500 |
| 7 | UBC 818 | CACACACACACACACAG | 47.1 | 3 | 1400-2500 |
| 8 | UBC 830 | TGTGTGTGTGTGTGTGG | 51.1 | 2 | 800-1500 |
| 9 | UBC 834 | AGAGAGAGAGAGAGAGYT | 40.4 | 2 | 800-1100 |
| 10 | UBC 835 | AGAGAGAGAGAGAGAGC | 41.8 | 4 | 850-1500 |
| 11 | UBC 840 | GAGAGAGAGAGAGAGAYT | 40.8 | 3 | 1100-1500 |
| 12 | UBC 841 | GAGAGAGAGAGAGAGAYC | 41 | 6 | 700-1700 |
| 13 | UBC 844 | CTCTCTCTCTCTCTCTRC | 41.5 | 1 | 1400 |
| 14 | UBC 848 | CACACACACACACACARG | 50.5 | 2 | 900-1450 |
| 15 | UBC 850 | GTGTGTGTGTGTGTGTYC | 48 | 1 | 1100 |
| 16 | UBC 873 | GACAGACAGACAGACA | 40 | 6 | 700-2500 |
| 17 | UBC 888 | BDBCACACACACACACA | 47.3 | 4 | 1000-1600 |

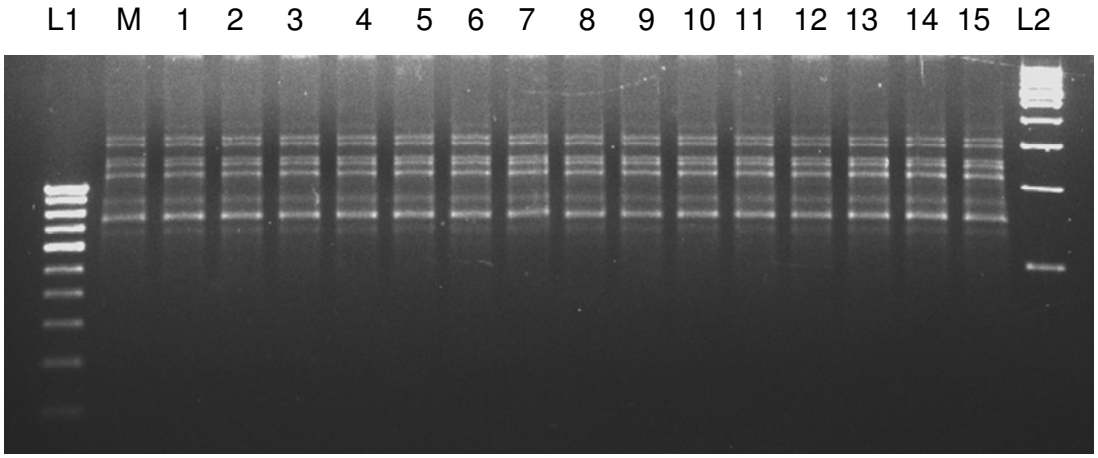


**Figure 2.** ISSR products generated from 15 *in vitro* regenerated plants and mother plants of *Guadua angustifolia* amplified with primer UBC 808 showing monomorphic pattern. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1 to 15 represent *in vitro* raised clones of *G. angustifolia* and Lane L2 represents 500-bp ladder.

Earlier, Das and Pal (2005) established the clonal fidelity of regenerants of *Bambusa tulda* and *Bambusa balcooa* using only four markers to assess their genetic uniformity among the regenerants. Later, Negi and Saxena (2010) employed 15 ISSR markers to validate the clonal fidelity of *in vitro* raised *B. balcooa* plantlets through the axillary bud proliferation. However, there is no report available on the comparative genetic stability of regenerants and mother plant of *G. angustifolia* by using RAPD and ISSR markers. In the present study, we did not find any polymorphism during the RAPD analysis of *in vitro* raised clones (Figure 1). This is consistent with the absence of genetic variations observed during micropropagation of *Pinus thunbergii* (Goto et al., 1998), turmeric (Salvi et al., 2001), Lilium (Varshney et al., 2001), *B. balcooa* and *B. tulda* (Das and Pal, 2005), *Dendrocalamus hamiltonii* (Agnihotri et al., 2009) and Gerbera (Bhatia et al., 2011), analyzed using RAPD markers. We screened 27 ISSR primers, however only 17 ISSR primers produced 61 distinct and scorable bands in the size range of 300 to

2500 bp. In addition, the banding profiles from micropropagated plants were monomorphic and similar to those of mother plant (Figure 2). Similar results have been reported in almond (Martin et al., 2004), banana (Lakshmanan et al., 2007), *Swertia chirayita* (Joshi and Dhawan, 2007), *Crataeva magna* (Bopana and Saxena, 2009), *B. balcooa* (Negi and Saxena, 2010), *Bambusa nutans* (Negi and Saxena, 2011), gerbera (Bhatia et al., 2011). Moreover, the common practice to use AFLP markers to identify clonal fidelity of plants (Mehta et al., 2010) requires state-of-art set up and expensive reagents. In our experience, AFLP method requires 10 times more investment for instruments and 5 times more disbursement for reagents compared to RAPD or ISSR method. Thus, the RAPD and ISSR primers allow simple and cost-effective method to (1) test clonal fidelity and (2) amplify different regions of genomes. This will provide us with better chances to identify the genetic variations in *in vitro* raised *Guadua* clones.

## Conclusion

In summary, we have confirmed the true nature of the *in vitro* raised clones of *G. angustifolia* Kunth using DNA based RAPD and ISSR markers as we did not detect any variability in the tissue culture raised plantlets. Thus, this will allow us to employ the axillary bud proliferation method for the commercial multiplication of *Guadua* without any risk of genetic instability.

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# Identification and elimination of bacterial contamination during *in vitro* propagation of *Guadua angustifolia* Kunth

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## ABSTRACT

**Background:** *Guadua angustifolia* Kunth is a very important bamboo species with significant utility in pharmaceutical, paper, charcoal, and construction industries. Microbial contamination is a major problem encountered during establishment of *in vitro* cultures of *Guadua*. **Objective:** This study has been designed to analyze the identity of contaminating bacteria and to develop the strategy to eliminate them during micropropagation of *Guadua*. **Materials and Methods:** We isolated and consequently analyzed partial sequence analysis of the 16S rRNA gene to identify two contaminating bacteria as (1) *Pantoea agglomerans* and (2) *Pantoea ananatis.* In addition, we also performed antibiotic sensitivity testing on these bacterial isolates. **Results:** We identified kanamycin and streptomycin sulfate as potentially useful antibiotics in eliminating the contaminating bacteria. We grew shoots on multiplication medium containing BAP (2 mg/l) and adenine sulfate (10 mg/l) supplemented with kanamycin (10 μg/ml) for 10 days and transferred them to fresh medium without antibiotics and found that bacterial growth was inhibited. Moreover, we observed intensive formation of high-quality shoots. Streptomycin sulfate also inhibited bacterial growth but at higher concentration. We also demonstrated that shoots grown in streptomycin sulfate tended to be shorter and had yellow leaves. **Conclusion:** Thus, we have developed a novel strategy to identify and inhibit intriguing microbial contaminations of (1) *Pantoea agglomerans* and (2) *Pantoea ananatis* during establishment of *in vitro* cultures of *Guadua*. This would improve *in vitro* establishment of an important bamboo, *Guadua angustifolia* Kunth for large scale propagation.

**Key words:** 16S rRNA gene sequencing, bacterial contamination, *Guadua angustifolia* Kunth, *in vitro* propagation

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## INTRODUCTION

*Guadua angustifolia* Kunth is one of the three largest and most important bamboo species in the world. Bamboo tar oil, recovered as a secondary product during the carbonization process, has significant medicinal value due to its antibiotic and antioxidant activities. *Guadua* also have a great potential to fix atmospheric carbon dioxide. Due to its versatility, lightness, flexibility, endurance, hardness, strength, climatic adaptability, seismic-resistance, rapid growth, and easy handling, it is widely employed in pharmaceutical, paper, charcoal, and construction industries.

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Bamboo culms are traditionally harvested from natural forests, but overexploitation has led to rapid depletion of their natural vegetative strands. As a result, most of the area under tropical rain forests and biodiversity has vanished and millions of hectares have been transformed into pastures and croplands. Therefore, there is a great concern about the conservation of bamboo's natural populations and thus need to develop novel propagation methodologies for new plantations and re-establishment of cleared strands.[1] The traditional propagation method by "offsets" limits the number of propagules. Moreover, the use of nodal segments for propagation is cumbersome and labor intensive for large-scale establishment of bamboo plantations.[2] Due to profound difficulties in the conventional propagation of bamboos, it is imperative to adapt alternative methods for rapid multiplication, and therefore micropropagation offers a feasible alternative.

Our attempts to obtain aseptic *in vitro* cultures using explant of *Guadua* from glasshouse-grown plants and to optimize a micropropagation procedure were hindered by persistent appearance of bacterial contamination in the cultures. Bacterial contamination in tissue culture is well documented,[3] and the failure of surface sterilization procedures to produce aseptic cultures is a major problem with woody plants.[4] Different experimental procedures including chemical sterilization and antibiotics have been used at various levels of success to minimize or eliminate such contamination. However, the type, concentration, and duration of antibiotic treatment vary for different plant tissue cultures. Therefore, it is pertinent to optimize antibiotic treatment strategy before its use.[5,6]

The growth medium selected for *in vitro* propagation also serves as a good source of nutrients for microbial growth. These microbes further compete adversely with plants for nutrients.[7] The presence of microbes or latent infections in these plant cultures usually results in increased culture mortality, variable growth, tissue necrosis, reduced shoot proliferation, and reduced rooting.[8]

We observed bacterial contamination in micropropagation of *Guadua angustifolia*. The contaminants were evident at the culture establishment stage and resulted in the loss of plants when bacteria overgrew the explants. In the present study, we have characterized two bacteria from *Guadua* shoot cultures, and determined effects of various antibiotics on these bacteria without adversely affecting the health of *in vitro* grown plant material.

## MATERIALS AND METHODS

### Disinfestation procedure

We used nodal segments measuring 2-4 cm cm in length from 4-year-old potted plants for initiating aseptic cultures. Briefly, we subjected explants to repeated washings after removal of leaf sheaths. This would remove all the adhering dust particles and microbes from the surface. The explants were then cleaned with a liquid detergent (Tween 20-HIMEDIA, Mumbai, India) followed by treatment with a suitable fungicide (e.g., Bavistin, 0.2%). Under sterile conditions in a laminar air flow bench, these explants were sterilized with 70% ethanol (v/v) and soaked in 0.04% $HgCl_2$.

### Initiation of aseptic cultures

The sterilized explants were placed vertically in test tubes containing the MS (Murashige and Skoog's) medium[9] supplemented with BAP (6-Benzylaminopurine, 2mg/l), sucrose (2%), and agar (Murashige and Skoog 1962). The pH was adjusted to 5.7 prior to autoclaving. The cultures were incubated at a photosynthetic photon flux density (PPFD) of 70 ± 5 μmol/$m^2$/s from cool, white, fluorescent lamps at 25 ± 2°C. Moreover, the day length was maintained at 16 hours in a 24-hour light/dark cycle.

### Isolation and identification of bacteria

Bacterial growth appeared as a cloudy zone in the agar medium around the shoot base within 20 days invariably in all the cultures. The contaminating bacteria were isolated by placing material with loop from visibly contaminated culture directly on the LB medium. After incubation at 28°C for 24 hours, two types of colonies were observed. Pure cultures of these bacteria were obtained by picking up the colonies and streaking them onto the fresh medium. These cultures were further maintained in glycerol stock at −80°C.

### Antibiotic treatment of plants

The luria agar plates containing different antibiotics like kanamycin, carbenicillin, ampicillin, rifampicin, and streptomycin sulfate were inoculated with isolated bacterial contaminants for antibiotic sensitivity screening.

We selected two antibiotics, kanamycin, and streptomycin sulfate, on the basis of their effectiveness in antibiotic sensitivity testing. To test their effectiveness in eliminating bacterial contamination, the antibiotics were added to the multiplication medium, i.e., the liquid MS medium containing BAP (2 mg/l) and adenine sulfate (10 mg/l) in the following dosages: 0, 5, 10, 15, 25, 40, 50 g/ml alone and in combinations. Contaminated explants were then dipped in this medium for 10 days. Controls (plant tissue grown in the multiplication medium without antibiotics) were also included with each experiment. After 10 days of the antibiotic treatment, the physical conditions of plants were noted again and then placed in the liquid multiplication medium without any antibiotic. Shoots with no detectable signs of bacterial contamination were individually transferred onto the fresh medium without antibiotics and subcultured every 3 weeks. Growth rate and plant appearance were monitored to determine whether the antibiotics had any phytotoxic effects on plants during the multiplication and rooting phase.

### 16S rRNA gene sequencing

We isolated bacterial DNA from pure culture and performed PCR amplification of almost the entire length of 16S rRNA gene fragment. We used following primers 5'-AGAGCTTTGATCATGGCTCAGA-3' and 5'-GTTACCTTGTTACGACTT-3' to amplify 8 to28 and 1493 to 1510 parts of 16S rRNA gene of *Escherichia coli* and are useful for amplifying the 16S rRNA gene from various kinds of bacteria. The PCR was performed and analyzed on an agarose gel as described earlier.[10]

The 16S rRNA gene of bacteria was further sequenced to analyze its identity. Briefly, the amplified 16S rRNA gene was purified from the agarose gel using a Nucleospin Extract II kit. The PCR-purified product was directly used for nucleotide sequencing of the gene by using a Big Dye$^R$ Terminator Cycle sequencing kit (Applied Biosystems). To identify bacteria, preliminary searches in the NCBI database were performed with BLASTIN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA).

## RESULTS AND DISCUSSION

The bacterial contamination encountered during *in vitro* propagation of plants is a major bottleneck which obstructs successful experimentation and establishment of aseptic cultures. Serious attempts have been made to develop procedures for eliminating these bacterial contaminants through (1) rigorous manipulation of environmental and nutritional factors or (2) treatment with antibiotics. [11] The association of bacteria with *in vitro* cultures of different crop plants, like watermelon, grape, banana, papaya, and capsicum has been encountered. This has been the cause of decline in the performance of cultures, degeneration of long-term maintained stocks, and lack of reproducibility of tissue culture protocols.[12-15]

We utilized 16S rRNA gene sequence analysis to identify bacterial contaminants [Figure 1] in *Guadua angustifolia* Kunth**.** These contaminants were found to be highly similar to *Pantoea agglomerans* (NCBI # FR872702) and *Pantoea ananatis* (NCBI # FR872704). Both bacteria are gram negative and closely related.[16,17] *P. ananatis* is a common epiphyte. It infects both monocotyledonous and dicotyledonous plants. It also occurs endophytically in hosts where it has been reported to cause disease symptoms.

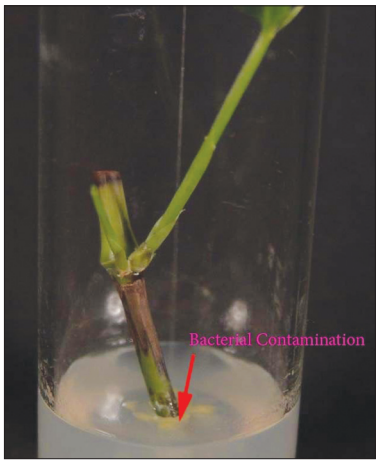


**Figure 1:** Bacterial contamination in the region around shoot base in the agar medium.The bacteria appeared as creamish white growth around the base of shoots in the agar gelled medium after 20 days of inoculation

Apart from being associated with plants as an epiphyte, pathogen, or symbiont, it also occupies diverse and unusual ecological niches where it may function as a saprophyte. *P. agglomerans* is known to be an opportunistic pathogen in the immunocompromised, causing wound, blood, and urinary tract infections. It is commonly isolated from plant surfaces, seeds, fruits (namely mandarin oranges), and animal or human feces.

Many bacteria grow slowly or not at all in media used in plant culture, thus escaping detection until considerable time and materials have been invested.[18] The ideal approach is to use antibacterial substances (e.g., antibiotics) but it has met with varying degrees of success.[3,19] In many cases, antibiotics have been found to be phytotoxic at high concentrations enough to destroy all contaminants.[3,20] The lack of descriptive information and antibiotic susceptibilities of a large number of plant-associated bacteria further complicate the use of antibiotics.[21] Therefore, the characterization and identification of plant-associated bacteria can lead to more successful antibacterial therapies.[19]

Our antibiotic sensitivity testing revealed kanamycin and streptomycin sulfate as the most effective antibiotic against the contaminating bacteria [Table 1]. The kanamycin was least phytotoxic during micropropogation of *G. angustifolia*. The shoot tips were grown for 10 days on the multiplication medium containing the kanamycin (10 μg/ml). The addition of kanamycin grossly inhibited the bacterial growth while allowing the formation of high-quality *Guadua* shoots [Figure 2]. In contrast, streptomycin was effective at reducing bacterial growth in tissue culture at higher concentrations (15 μg/ml). Moreover, the shoot number and the quality of *Guadua* were also reduced. Such inhibition of shoot growth by streptomycin has also been noted during micropropagation of *Pelargonium*.[22]

Kanamycin interacts with the 30S subunit of prokaryotic ribosomes. It induces substantial amount of mistranslation and indirectly inhibits translocation during protein synthesis. Streptomycin binds to the S12 protein of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This prevents initiation of protein synthesis and leads to death of microbial cells. It may also inhibit protein synthesis in chloroplasts and mitochondria in plant tissues, and thus resulting in small and yellow leaves.

Traditionally, combinations of antibiotics have been used against bacterial contaminants in plant tissue culture.[3,23] The combinations of two or more antibiotics for eliminating bacterial contaminants are very well recommended.[4,22] However, we interestingly found kanamycin (10 μg/ml)

**Table 1: The effect of various antibiotics on the growth of bacteria and *Guadua angustifolia* Kunth**

| Antibiotic | Concentration (µg/ml) | Removal of bacteria | Health status of shoots |
|---|---|---|---|
| Streptomycin sulfate | 0 | − | Overgrowth of bacteria inhibited shoot survival |
| | 5 | + | Overgrowth of bacteria inhibited shoot survival |
| | 10 | ++ | Shoots were fresh, green, and healthy |
| | 15 | +++ | Shoots were yellowish green with small leaves |
| | 25 | +++ | Shoot necrosis |
| | 40 | +++ | Shoot necrosis |
| | 50 | +++ | Shoot necrosis |
| Kanamycin | 0 | − | Over growth of bacteria inhibited shoot survival |
| | 5 | ++ | Overgrowth of bacteria inhibited shoot survival |
| | 10 | +++ | Shoots were fresh, healthy, and green |
| | 15 | +++ | Shoots were yellowish green |
| | 25 | +++ | Shoot necrosis |
| | 40 | +++ | Shoot necrosis |
| | 50 | +++ | Shoot necrosis |

"+" sign denotes a positive response to some extent; "++" sign denotes a positive response to moderate extent; "+++" sign denotes a complete positive response; "−" sign denotes no response

**Figure 2:** The growth of healthy *G. angustifolia* shoots in the multiplication medium after treatment with kanamycin. The treatment of shoots with kanamycin (10 µg/ml) grossly inhibited the bacterial growth without affecting their quality and shoot numbers

**Figure 3:** Acclimatized plants of *G. angustifolia.* Plantlets were transferred to plastic pots containing sand in polytunnels and covered with jars to maintain high relative humidity. After 1 month of hardening, these plants demonstrated 90% survival when transferred to pots containing 1:1:1 mixture of soil, sand and manure

as very effective in eliminating bacterial contaminants with least phytotoxicity. Such shoots with no detectable signs of bacterial contamination were transferred onto the fresh multiplication medium without antibiotic after every 3 weeks. The multiplication rate of shoots treated with antibiotic was similar to that of healthy plants. These shoots were able to produce healthy roots in the same multiplication medium without addition of auxin. These plants were successfully hardened under green house conditions [Figure 3].

## CONCLUSION

The common problem of bacterial growth around *in vitro* shoots in *Guadua angustifolia* tissue culture is due to (1) *Pantoea agglomerans* and (2) *Pantoea ananatis*. This can be resolved through treatment of *G. angustifolia* shoots with kanamycin for 10 days. No phytotoxicity appeared when shoots were treated with kanamycin (10 µg/ml) and the multiplication rate of treated *G. angustifolia* shoots was found to be similar to that of healthy plants. Streptomycin sulfate, at higher concentration, also inhibited bacterial growth during micropropagation of *G. angustifolia*. In addition, shoots grown in streptomycin sulfate tended to be shorter and have stunted leaves. Thus, our study provides us with a technique to identify and resolve bacterial contamination of (1) *Pantoea agglomerans* and (2) *Pantoea ananatis* during *in vitro* culture of *G. angustifolia*.

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