‘Establishment of Culture Systems for the Production of Clonal Planting Materials of *Cinnamomum tamala* (Buch-Ham) T. Nees. & Eberm.’

By

Mrs. Madhabi Saha Deb

A THESIS SUBMITTED

IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BOTANY

OF

DEPARTMENT OF BOTANY

NAGALAND UNIVERSITY, LUMAMI - 798 627

NAGALAND, INDIA

2012

CONTENTS

Nagaland University

(A Central University Estd. By the Act of Parliament No. 35 of 1989)
Lumami 798 627, Nagaland, India

November, 2012

DECLARATION

I, Mrs. Madhabi Saha Deb, bearing Ph. D. registration number 410/2011 (December 06, 2007) hereby declare that, the subject matter of my thesis entitled 'Establishment of Culture Systems for the Production of Clonal Planting Materials of *Cinnamomum tamala* (Buch-Ham) T. Nees. Eberm.' is the record of original work done by me, and that the contents of this thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. The thesis has not been submitted by me for any research degree in any other University/Institute.

This thesis is being submitted to the Nagaland University for the degree of 'Doctor of Philosophy' in Botany.

Madhabi S. Deb.
12/11/12
(Mrs. Madhabi S. Deb)
Candidate

12/11/12
(Prof. N. S. Jamir)
Supervisor
Prof. N.S. Jamir
Department of Botany
Nagaland University
Hqs.: Lumami Nagaland

Chilla Roy Deb
12/11/12
Head
Department of Botany
Head
Department of Botany
Nagaland University
Hqs. Lumami.

Acknowledgement

My sincere gratitude to my Supervisor Prof. N. S. Jamir, Department of Botany, Nagaland University, Lumami, who guided and motivated me with his valuable suggestions and continuous encouragement to pursue my research work despite my flaws. "*Without your help and unending encouragement I would never have made it this far. Thank you very much, Sir*".

I would also like to thank all those who have helped me and guided me in various forms during my endeavours:

Honourable Vice Chancellor, Nagaland University for giving me the opportunity to complete my research works in this University and also for awarding me UGC's Non-NET fellowship during my research work.

The Head, Department of Botany, Nagaland University for all the necessary facilities for my research work and helping in various ways.

Prof. S. K. Chaturvedi, Dr. Limasenla, Dr. Talijungla, Dr. S. Kumar, Dr. N. Puro, faculties, Department of Botany for their encouragement and helps.

All non-teaching staff of the Department for their helps.

All my Lab mates and Research Scholars of the Department, who helped me in various ways and for their continuing friendship and understanding.

Miss. T. Arenmongla and her family for allowing me to collect experimental materials from the tree they maintained.

My loving daughters (Chandrima and Sreejaya) for their understanding, co-operation and help, my loving husband Dr. Chitta Ranjan Deb, Associate Professor, Department of Botany for his continuous help, guidance and encouragement during my research works.

All my family members, my parents for their unending encouragement and continuous prayer and support throughout my research period.

Above all I thank our all mighty God for the grace and guidance in completing this work.

Madhabi S. Deb.

(Madhabi S. Deb)

List of Figures

Figure 9: Stages involved on *in vitro* induction of morphogenetic response from the cultured nodal segments of *C. talmala*. **a**. Cultured nodal explants showing swelling of node and invocation of meristemetic response, **b**. Nodal explants showing a shoot bud formation, **c**. Multiple shoot bud formation.

Figure 10: Callus induction from different explants of *C. tamala* and shoot bud formation. **a.** callus formed throughout the leaf surface, **b.** Callus proliferation and induction of shoot buds from leaf raised callus, **c.** Callus formed from cultured embryo, **d**. callus formed from cultured nodal explants, **e**. Callus proliferation and shoot bud induction and f. Shoot buds developed from the cultured callus.

Figure 11: Effect of different concentrations of sucrose on regeneration of plantlets and culture proliferation of *Cinnamomum tamala*.

Figure 12: a. Shoots buds cultured on regeneration medium developed on initiation medium from different explants, **b**. Regenerated plants on regeneration medium, **c**. A plant showing formation of rooting.

Figure 13: a. Rooted plants transferred to potting mix established in the poly house, **b**. A transplant established in the natural habitat.

List of Tables

Chapter - 1

Introduction

Human kinds have always been fascinated by the diversity of life. Biodiversity is the results of the evolutionary plasticity of living organisms which has increased geometrically through perhaps 2.5 billion years, proliferating by trial and error, controlled by natural selection, filling almost every one of the habitable ecological niches created in a likewise evolving world environment. The diversity of these natural habitats has expanded over a period of time through natural selection and also through introductions. Biodiversity, in wild and domesticated forms, is the source for most humanity food, medicine, clothing and housing, most of the cultural diversity, and most of the intellectual and spiritual inspirations. Biodiversity includes diversity of form right from molecular level through individual, organisms, populations, communities, ecosystems, landscapes and biosphere. It exists at three different levels. These are:

1) Species diversity, which embraces the variety of living organisms on earth.

2) Genetic diversity, which is concerned with the variation in genes within a particular species

3) Ecosystem diversity, which is related to the variety of habitats.

The world harbors an estimated over 10 million different types of organisms including plants, micro-organisms and animals, out of which about 1.4 million species are that of plants including algae, fungi, mosses and higher plants (Myers *et al.*,2000). The recent report of IUCN brings out a list of 34 biodiversity hotspot regions of the world which indicates an alarming situation the world is faced with, in terms of biodiversity resources *vis-a-vis* future of mankind. Biodiversity hotspots are geographical regions which are extremely rich in species, have high endemism, and are under constant threat. Out of the estimated total global biodiversity of 10 million species in the world, India is one of the 17 mega-diversity in the world. The North-Eastern region, Western Ghats, North-Western, Eastern Himalayas and Andaman and Nicobar Islands are home for rich floral and faunal diversity. India is a tropical country with a tremendous heterogeneity of environments ranging from tropical rain forest of Andaman and Arunachal Pradesh to the hot deserts of Rajasthan and cold desert of Ladakh. It lies at the junction of three biogeographical provinces of Africa, temperate Eurasia and the Orient.

Plants as a Source of Medicines

Nature has provided a rich store house of herbal remedies to cure all mankind's ill. Throughout the world people have utilized several thousands of plants and plant products as cure

for human ailments. In the plant kingdom, almost all plants are medicinal and the application of medicinal plants especially in traditional medicine is well recognized (Chaudhary and Tariq, 2006). In the developing countries, drugs are not only expensive, but also have many side effects during treatment for any disorders that is why in the present era it is being emphasized to search medicinally valuable plants.

India has one oldest, richest and diverse cultural traditions associated with the use of plants and herbs for human, livestock and plant health. Many of the ingredients of Indian cooking which have been handed down from ages contain medicinal properties (Chakraborty and Das, 2010). In India, the '*Ayurvedic System of Medicine*' has been in use for more than 3000 years. '***Charaka & Susruta***' two of the earliest Indian authors had sufficient knowledge of the properties of the Indian medicinal plants. **'*Charaka Samhita*' and '*Susruta Samhita*'** which are their medical works are one of the esteemed treasures of literature of indigenous medicine today (Deb *et al.*, 2009).

Plants have been used in the traditional health care system from the time immemorial, particularly among tribal communities. The World Health Organization (WHO) has listed over 20,000 medicinal plants globally (Laloo *et al*., 2006); India's contribution is ~20%. According to WHO estimate, about 80% of the population in the developing countries depends directly on plants for their sole source of medicine (Deb *et al.*, 2009). In India, about 2000 drugs used are of plant origin (Dikshit, 1999). Though a vast ethno botanical knowledge exists in India from ancient times, very few plants used by locals for medicine are subjected to scientific investigation. The need for conservation of medicinal plants and traditional knowledge,

particularly in developing countries like India, taking into account the socio-cultural and economic conditions is urgent (Misra, 1999).

Plants and Spices

In addition to medicinal value of plants, they also provides us with many other useful products such as dyes, tannins, waxes, resins, flavoring agents, rubber, spices etc. Indian spices are well-known all over the world for their taste and strong aromatic flavor. There are around 80 types of species grown throughout the world but India alone produces about 50 types of them (Rathore and Shekhawat, 2008). The history of spice is almost as old as human civilization. It is a history of lands discovered, empire built and brought down, wars won and lost, treaties signed and flouted, flavors sought and offered and the rise and fall of different religions practices and beliefs. Spices were among the most valuable items of trade in ancient and medieval times. The use of spices spread through the Middle East to the Eastern Mediterranean and Europe. Spices from China, Indonesia, India and Sri Lanka were originally transported overland by donkey or camel caravans. Arab middlemen controlled the spice trade, until European explorers discovered a sea route to India and other spice producing countries in the East (Hadacek, 2002).

The spices that India offers in abundance are *pepper, ginger, turmeric, chilli, cardamom, celery, fennel, cumin, clove, coriander,* ***cinnamon****, ajwain,* ***cassia****, nutmeg, mace* etc. Each of these spices has its own flavor, medicinal value and other interesting facts to go with it. In fact, the very commonly used word '***aroma***' is the ancient Greek work for spice. Spices can improve the palatability and the appeal of dull diets or spoiled food. Piquant flavors stimulate salivation and promote digestion. Spices may be used ground or whole, fried or roasted, dry or as paste, at

the end or in the beginning of the cooking, alone or in combination (Andrews *et al.*, 1995; Rathore and Shekhawat, 2008).

Indian grocery stores offer almost all spices and grains that are necessary for Indian cooking. Indian spices can be mixed in specific amounts to make spice mixtures used in daily cooking, for example 'GARAM MASALA'. Spices add zest to food, enhance the taste, and delight the gourmet. Spices can also be used as preservatives, aphrodisiacs and in traditional medicines. Spices are derived from different parts of plants e.g., flowers, fruits, leaves, seeds, rhizomes, roots, bark etc. India produces almost all the spices, largely owing to its varying climate and soil conditions. The classic Indian curry often combines the following spices – *coriander, **cinnamon bark and leaf (tejpat)**, turmeric, cumin, ginger, garlic* as well as other spices. Spices are taken from the parts of the plant richest in flavor like bark, leaf, stem, flower buds, seeds etc. Spices and herbs are good not only for our taste buds but also for our health. *Cinnamomum tamala* is also the source of calcium, iron, vitamin B, C, carotene and other antioxidants.

Importance of Seed Biology

Inside each seed is a living plant embryo that even in a state of dormancy breathes through the exchange of gases and is consistently undergoing metabolic (aging) process. The natural lifespan of a seed is influenced by several factors including: permeability of the seed coat, dormancy, seed physiology and storage environment. Seeds of many of our native plants and weedy alien species have dormant embryos and hard seed coats, a condition that retards germination and consequently enhance longevity. The presence and degree of seed dormancy

and subsequent metabolic rates varies considerably between species and thus influences their lifespan. For most species from temperate and arid climates reducing and maintaining a low seed moisture content, storing seeds at low to moderate temperatures, and taking precautions not to damage seeds during cleaning and handling, slows down the metabolic process and thereby increases their longevity in storage.

Seeds are generally categorized into three types:

1. **Orthodox**: Seeds that can be dried, without damage, to low moisture contents, usually much lower than those they would normally achieve in nature. Their longevity increases with reductions in both moisture content and temperature over a wide range of storage environments.

2. **Recalcitrant**: Seeds that do not survive drying to any large degree, and are thus not amenable to long term storage.

3. **Intermediate**: Seeds that are more tolerant of desiccation than recalcitrant, though that tolerance is much more limited than is the case with orthodox seeds, and they generally lose viability more rapidly at low temperature.

Recalcitrant seeds are not only desiccation-sensitive, but also metabolically active. In contrast, orthodox seeds, owing to their dry state, are metabolically quiescent (Berjak, 2005). One can estimate a species' natural potential for storage tolerance by:

1. ***Seed size***: Large seeds often have high moisture content and are generally recalcitrant in their storage behavior.

2. ***Climate and habitat conditions in which the species grow***: Seeds from plants adapted to tropical or riparian habitats, due to a semi to permanent water source and/or consistently mild and reliable growing conditions, may not require long term seed viability for survival. Conversely, plants from desert, temperate climates, where environmental conditions suitable for germination are often infrequent, are more likely to produce seeds capable of surviving for long periods.

3. ***Seed physiology***: A heavy impervious seed coat even on large seeds, as is often found on desert legumes and lupines, promotes long-term seed viability.

4. ***Life cycle***: Annual and perennials are more dependent on a persistent soil seed bank than woody and long-lived shrub and tree species.

5. ***Ecological association***: Plants that are early succession colonizer species that may occur only after disturbances and species that depend on other plants for their development must maintain viability until a suitable host plant is available.

Recruitment of tree species on the forest floor or in the field is governed by various factors including the seed traits and microhabitat conditions (Kitajima, 2007). Likewise, germination of seeds in nature is strongly influenced by internal as well as external environmental factors (Baskin and Baskin, 1998). The germinating seeds and seedlings are most vulnerable to predation, desiccation, developmental stage of embryos, competition and damage as the seeds and seedlings constitute important resource for the herbivores and pathogen. Seedling predation and disturbance in the form of trampling and microhabitat alternation have direct implications for recruitment and multiplication of species. Consistency in microhabitat

condition, right developmental stage of the embryos in the harvested seeds, right desiccation of the seeds and duration of post harvest storage of seeds enhances seedling survival and plantlet growth. One of the important microhabitat factors in the forest floor is light regime which is governed by the tree canopy cover. Tree species vary considerably in terms of light requirement at seedling stage (Pacala *et al.*, 1994; Davis, 2001). Germinating seeds, depending upon their state such as inherent properties, developmental stage, reserve food materials, moisture content, nature of pericarp, represents their own microenvironments which have rarely been studied. In some specie even if one of the factors are not favorable, though there may be initial seed germination, seedlings fails to establish in the seed bed and subsequently in the field.

Many forests have a high number of species with recalcitrant seeds, or seeds sensitive to desiccation. The intolerance to desiccation leads to difficulties in conserving those seeds (Barbedo and Cicero, 2000). Many programmes that involve the rational and economic use of the tree species are hampered due to the lack of storage methods of recalcitrant seeds for longer periods. The methods in use preserve such seeds for periods varying from days to months. Thus, inclusion of several species in those programmes is limited. Identification and conservation of recalcitrant seeds as well as studies increasing their tolerance to desiccation were performed and pointed out respectively by several authors (Berjak *et al.,* 1990; Pammenter and Berjak, 1999; Pammenter *et al.*, 1991; Neves, 1994). In general, there is an agreement about the desiccation intolerance of recalcitrant seeds and their short longevity. Moreover, differences between recalcitrant and orthodox seeds at the end of the process of seed formation are well known. The orthodox seeds undergo a nearly essential process of desiccation, at the end of maturation. This process allows these seeds to change their metabolism from development to germinate

(Kermode, 1990). These changes are not observed in recalcitrant seeds due to several factors including hormonal balance, protein and sugar contents and water physical properties, among others (Kermode, 1990; Barbedo and Filho, 1998).

The available methods of seed drying are not suitable for recalcitrant seeds, new approaches on seed germination and storage should be matter of investigation. Although it is not possible to define unequivocally '**critical moisture content**' for viability loss in recalcitrant seeds because they show differential response to dehydration at different drying rates, it is necessary to maintain their water level above critical levels (Pammenter *et al.*, 1998). Under these conditions, however, seeds may initiate germination and or undergo microorganism infection, which could lead to seed deterioration. High humidity favors the initial steps of seeds germination processes causing damage to seeds if water is not supplied in sufficient. Consequently, storage treatments maintaining high moisture of the seeds are often associated with low temperatures (Cunha *et al.*, 1995). Nevertheless, many of these seeds have little tolerance to cold conditions. Therefore, studies on these factors will give a better understanding on seed storage, seed germination and seedling establishment of the species produces recalcitrant seeds.

Plant Tissue Culture, Mass Multiplication and Conservation of Economically Important Plants

Indian subcontinent is a home for large number of economically important plants but the plants of this regions are getting depleted due to various anthropogenic activities such as removal of forest cover, '***Slash and Burn'/'Jhum'*** cultivation and other developmental activities like

industrialization and unplanned human activities. The natural vegetation is under great threat, causing depletion in the population of many economically important plants. Therefore, it is necessary to develop protocols for mass multiplication and *in vitro* conservation of these threatened plant species. Plant tissue culture technique is a boon in the studies of biosynthesis of secondary metabolites and provides an efficient means of producing many economically important plant products. Plant tissue culture is a technique of growing plant cells, tissue and organs in an artificial prepared liquid medium or semi-solid medium under aseptic condition.

One of the most important problems that stands on the way of propagating is the difficulty of raising the plant through seeds, because when the seeds are shed from the plant the embryos inside is undifferentiated, but plant tissue culture techniques has proved to be a great boon to combat these problems. Through this technique, the vegetative parts can be induced to form callus on a defined nutritive medium containing appropriate quantity of auxins and cytokinins and can be differentiated into large number of plants or as a direct organogenesis within a short period of time. In nature, the clonal propagation takes place through bulbs, corms, suckers, cuttings, offshoots, etc., but they are unable to meet the demand of *elite* clones market. Therefore, plant tissue culture technique has become extremely significant in agriculture which has been used in various fields such as hybridization, variety development, maintaining pathogen free plants and also for rapid clonal propagation and conserve whose populations are facing threat of extinction. It potentially provides many production advantages like-

1. a large number of plantlets can be produced inexpensively.

2. quick and easy scale-up can be achieved.

3. Short to medium-term germplasm storage via slow growth of cultures.

Many plant species have been propagated successfully through plant tissue culture technique particularly the threatened orchid species and reintroduced into the wild ameliorating their status in nature. Different explants sources like seeds, foliar explants, rhizome, roots, inflorescence, etc. have been used for *in vitro* propagation of different species of plants by many workers for conservation programme. Following are some of the works done by various workers: *Aerides multiflora* Roxb. (seeds- Katiyar *et al.*, 1987; foliar segment- Vij and Pathak, 1990; aerial roots- Vij- 1993); *Arachnis labrosa* (seeds- Temjensangba and Deb, 2005a; foliar segments- Deb and Temjensangba, 2007a; aerial roots- Deb and Temjensangba, 2006a); *Cleisostoma racemiferum* (seeds and leaf- Temjensangba and Deb, 2005b, c, 2006; aerial roots- Deb and Temjensangba, 2005); *Coelogyne porrecta* Lindl. (seeds- Abdul Karim and Hairani, 1990); *C. suaveolens* Lindl. (seeds- Sungkumlong and Deb, 2008, leaf- Deb and Sungkumlong, 2010); *Cymbidium elegans* Lindl. (seeds- Raghuvanshi *et al.*, 1991); *C. iridioides* D. Don. (seed- Pongener and Deb, 2009; nodal segment- Pongener and Deb, 2011a); *C. aloifolium* (seed- Pongener and Deb, 2011b); *Dendrobium chrysanthum* Wall. ex Lindl. (seeds- Raghuvanshi *et al.*, 1986); *D. fimbriatum* var. *oculatum* Hk. f. (D. Don.) (seeds- Devi *et al.*, 1990); *D. nobile* Lidl. (seeds- Raghuvanshi *et al.*, 1986); *D. primulinum* Lindl. (seed- Deb and Sungkumlong, 2009); *Eulophia alta* (L.) Fawcett & Rendle (seed- Johnson *et al.*, 2007); *E. hormusjii* Duth. (rhizome segments- Vij *et. al.*, 1989); *Haemaria discolor* (Mandarin: Xue-ye-lan or Cai-ye-lan) (seeds- Shiau *et al.*, 2005); *Luisia teretifolia* Gaud. (foliar segments- Vij and Pathak, 1990); *Malaxis khasiana* Soland ex. Swartz (seeds- Deb and Temjensangba, 2006b); *Rhynchostylis gigantia* (immature seeds- Li and Xu, 2009); *Rhynchostylis retusa* (L.) Bl. (seeds- Nath *et al.*,

1991; aerial roots- Chaturvedi and Sharma, 1986; Sood and Vij, 1986; foliar segments- Vij and Pathak, 1990); *Taenia latifolia* Lindl. (seed- Deb and Sungkumlong, 2008; pseudobulb- Sungkumlong and Deb, 2009; leaf- Deb and Sungkumlong, 2010); *Vanda cristata* Lindl. (foliar segments- Vij and Pathak, 1990); *V. testaceae* (Lindl.) Reichb. f. (foliar segments- Vij and Pathak, 1990); *Vanda* Kasem's Delight 'Tom Boykin (aerial roots- Vij and Sharma, 1997), *Acacia confusa* Merr (leaf- Arumugam *et al.,* 2009), *Solanum nigrum* (L.) (leaf- Sridhar and Naidu, 2011), *Stevia rebaudiana* (leaf- Preethi *et al.,* 2011, *Sphaeranthus indicus* L. (leaf- Yarra *et al.,* 2010), *Laelia speciosa* (leaf- Ochoa *et al.,* 2010).

A wide range of endangered plants have now been successfully propagated using *in vitro* techniques. There are many reports on *in vitro* multiplication of different types of orchids. Different workers have reported regeneration of plants in cultures using different explant sources like shoots, roots, seeds, axillary buds, pseudobulbs and leaves.

Seed/Embryo Culture

The technique of seed culture is variously referred to as embryo culture, which ensures better germination frequency and favors the production of virus free seedlings at a faster rate. For establishment of culture from the seed/embryos, it is important to identify the right development stage of the embryos. Seeds of many plant species undergo dormancy after maturation and at times fail to give response under *in vitro* condition. Because of this for many species it is necessary to harvest the immature seeds/embryos for successful culture establishment. The earliest stage at which the embryos can be cultured successfully varies with the orchid genotype and the local conditions. Very young ovules do not form suitable explants in

orchids because the embryo sac development is a post pollination phenomenon and fertilization a prerequisite for obtaining seedlings. However, as the ovules can be used for raising cultures immediately after fertilization, the importance of information on time interval between pollination and fertilization has often been stressed (Valmayor and Sagawa, 1967). *Doritis* ovules from pollinated ovaries germinated readily after getting fertilized *in vitro* (Yasugi, 1984) suggesting that fertilization is a pre-requisite for germination. Yam and Weatherhead (1988) also noted that immature embryo germinates better than the mature ones due to their distended testa cells and metabolically awakened embryos; they also lack dormancy or inhibitory factors. *Arachnis labrosa* and *Cleisostoma racemiferum* embryos obtained between 16 and 18, and 16 weeks after pollination (WAP) respectively (Temjensangba and Deb, 2005a, c, 2006); readily germinate but their germination frequency declines sharply, when obtained from beyond this window period. Likewise, in *Satyrium nepalense, Nephalaphyllum cordifolium, Phaius tankervilliae* and *Cymbidium* species, germination frequency shows sharp decline when the embryos are collected 3-4 weeks prior to fruit dehiscence.

Meristem Culture

The embryo culture produces a great deal of heterozygosity in their progeny due to cross pollination in many species. Because of this, it appears to be a disadvantageous proposition in cut-flower industry where pure lines of desired genotypes are preferred. This technique of using resident meristem (shoot-tips, axillary bud) has opened new vistas in micropropagation (Arditti and Ernst, 1993; Deb and Temjensangba, 2005, 2006a). Through this technique, up to 200,000 plants can be regenerated from a single resident meristem within a year. The source, genetic constitution and physiological age of the explants are however, some of the important factors for

regeneration. The juvenile tissues from greenhouse grown plants respond better than the mature ones grown outdoors. Generally, the proliferative loci get activated in the sub-epidermal cells and soon develop into somatic embryos and or shoot buds. Somatic embryogenesis is either direct or callus mediated development, and multiplication and differentiation of plantlets is influenced by the chemical stimulus present in the nutrient pool (Seeni and Latha, 1992; Vij and Pathak, 1990).

The advantages of leaf, nodal segments culture are apparent for more than one reason: they are easy to obtain, easier to disinfect, and their excision does not endanger the mother plant. Furthermore, as the regeneration occurs in the dermal cells, which is cytologically more stable, mass production of genetically uniform plant from this is within the realm of reality (Vij, 2002).

Different species exhibit specific needs in respect to nutritional requirement and treatment with plant growth regulators (PGRs) for their growth and development. So, no standard media formulation can be prescribed for all the species. Most commonly employed basal media for tissue culture are Knudson 'C' (1946) for orchids, Mitra *et al.* for orchids (Mitra *et al.*, 1976), Murashige and Skoog (MS) (1962) for different types of plants, Nitsch and Nitsch (1969), Vacin and Went (1949). The use of α-Naphthalene acetic acid (NAA) and one of the cytokinins like Benzyladenine (BA) and Kinetin (Kn) yields a rich crop of propagules. Similarly, in *Rhynchostylis retusa*, a synergistic action of Kn and indole 3-acetic acid (IAA) or NAA in peptone enriched medium favors enhanced production of PLBs; while yeast extract is obligatory for regeneration in *Aerides multiflorum, Papilionanthe teres* and *Satyrium nepalense* foliar cultures and peptone in those of *Vanda* (Vij, 2002).

Cinnamomum tamala (Buch.-Hum.) T. Nees. & Eberm. : A Valuable Multipurpose Tree

The genus *Cinnamomum* belonging to the family ***'Lauraceae***' comprises 270 species which occur naturally in Asia and Australia (Sharma and Nautiyal, 2011). They are evergreen trees and shrubs and most species are aromatic and many are economically important. About 20 species occurs in India (Anonymous, 1950). The etymology is derived from the Greek word '***Kinnamomon***' (meaning spice). The Greeks borrowed the word from the Phoenicians, indicating that they traded with the East from early times. The specific epithet '*tamala*' is after a local name of the plant in India. *Cinnamomum tamala* (Buch.-Hum.) T. Nees. & Eberm. **(Fig. 1)** is locally called as '*tejpat*' and also known as '*tejpatra*' in Sanskrit (Kirtikar and Basu, 1981). The species is known by many names in different languages e.g., **Arabicb** – *zarnab*, **Assamese** – *tez pat***, Bengali** – *tejpat, tezpat,* **Garo** - *tez-pat*, **Hindi** – *barahmi, dalchini, dalchunu, darchini, tajkalam, tajpat, talispatar, tomal pattae etc.*, **Kannada –** *dalchini, lavangadapatti,* **Sanskrit** – *ankusha, chhadana, coca, dala, ishtagandha, kalaskandah, patram, suranigandha, surasa, tamala, tejpatra* etc.

Cinnamomum tamala is a medium-sized tree found in India along with North-Western Himalayas, Sikkim, Assam, Mizoram, Meghalaya, Tripura and other places. The species also found in tropical and sub-tropical Asia, Australia, Pacific region and South Asia (Showkat *et al*., 2004). This evergreen species occurs as associated species in transitional evergreen broad leaf forest and is confined between sub-montane broad leaf ombrophilous forest (below 1000 m above MSL) and mid montane broad leaf ombrophilous forest up to 3000 m (Singh and Singh, 1992). According to Gaur (1999), *C. tamala* commonly occurs on moist-shady ravine slopes, often associated with Oak-Rhododendron forest, from 500 m to 2200 m MSL altitude in Himalaya.

Figure 1: A part of the *Cinnamomum tamala* tree showing mature and immature twigs.

The species is a perennial or small evergreen tree, attaining 8-12 m height and a girth of ~150 cm. Stem rough with gray-brown, soft wrinkled bark which produces mucilage. *C. tamala* has bisexual flowers. Flowers whitish, numerous, small, in axillary cymes **(Fig. 2a).** The plants produce flowers during April-May. The fruits are ellipsoidal drupe **(Fig. 2b)** and contain one seed in each fruit. The fruits require ~4 months for attaining maturity (present study). Ripe fruits are dark purple in color **(Fig. 2c).**

The leaves of *C. tamala* (*tajpat*) are widely used as a spice and also yield an essential oil on distillation. The essential oil of the leaves called '*tajpat oil'* is medicinally used as carminative, anti-flatulent, diuretic, and in cardiac disorders (Showkat *et al.*, 2004). '*Ayurveda*' describes the use of leaves of *tajpatra* in the treatment of ailments such as anorexia, bladder disorder, dryness of mouth, diarrhea, nausea etc. (Kapoor, 2000). It has hypoglyacemic and

hypolipidemic properties and but is commonly used in food industry, because of its special aroma (Chang and Cheng, 2002). The main constituents of *C. tamala* leaves are α-pinene, camphene, myrcene, limonene, eugenol, p-cymene, methyl eugenol, eugenol acetate and methyl ether of eugenol (Saino *et al.*, 2003).

Eugenol (4-hydroxy-3-methoxy allylbenzyme) is one the main constituents of cinnamon oil (Fischer and Dengler, 1990; Dighe *et al.*, 2005). Cinnamon bark oil possesses the delicate aroma of the spice and a sweet and pungent taste. Its major constituent is cinnamaldehyde but other minor components impart the characteristic odor and flavor. It is employed in the flavoring industry where it is used in meat and fast food seasoning, sauces and pickles, baked foods, confectionery, tobacco flavors and in dental and pharmaceutical preparations (FAO, 1995). *Tejpat* has been used as a natural food preservative for many fruit juice (Kapoor *et al.*, 2008).

Figure 2: a. A twig of *C. tamala* showing inflorescences, b. Immature green seeds, c. Mature seeds of *C. tamala*.

Its leaves and bark are aromatic and traded as a spice (Dhar *et al.*, 2002; Anonymous, 2006). Indian bay-leaves (trade name of the species) are closely related to cinnamon. These tough, three-veined leaves are very popular in Northern India, Bengal but are little known in

South and elsewhere. Its leaves and bark are aromatic and fragrant and therefore it is a common species in trade. It is regarded as carminative and used for treatment of diarrhea and colic pain. In Kashmir it is used as a substitute for betel leaves. The bark of *C. tamala* is coarser than the bark of *C. zeylanicum* and is one of the common adulterants of true *Cinnamon*. The essential oil from bark is pale yellow, and contains 70-80% cinnamic aldehyde. The leaf oil of *C. tamala* resembles cinnamon leaf oil. *C. tamala* is one of the three ingredients of '*trijalapatra*' with *C. zeylanicum* (dalchini) and *Elettaria cardamom*, mentioned by *Bhavaprakasa*. *Trijata* is commonly used in *Ayurvedic* pharmacy in *asava* and *arista* preparation to augment the fragrance and to promote the appetite and digestion. It has been reported the crude drug from unripe fruit of *C. tamala* is being sold under the name '*nagkesara*' in different parts of India (Vaidya, 1971).

Parts of *C. tamala* are also used in many *Ayurvedic* preparations e.g. *sudarshan choorna* and *chandraprabhavati*. The leaf extracts are used as clarifiers in dyeing procedures with myrobalans. Traditionally green dye has been extracted from its leaves (Gaur, 2008). Besides its high economic importance, this species provides excellent habitat for a large number of frugivorous birds and small mammals, which facilitate its regeneration in turn (Sharma *et al.*, 2009).

Owing to its high medicinal value and being an important ingredient of the spices the demand of *C. tamala* is increasing day by day and the species is being exploited from its natural pockets illegally (Samant *et al.*, 2001, Sharma and Nautiyal, 2009). Therefore, there is a need to raise high quality individuals in large scale to fulfill the increasing demand on the one hand and help the conservation of the species on the other. Although a lot of literatures was found about the pharmacological and chemical characteristics of *C. tamala* which are cited in the previous

sections, but very few works are done on physiological (Rawat *et al.*, 2009), micropropagation (Sharma and Nautiyal, 2009) and conservation and need further research.

Objectives of the Present Study

Studies on seed biology, the use of plant tissue culture technique are visualized for the conservation of rare/threatened/endangered species in mass multiplication of the species with extremely reduced populations. A wide range of economically important, threatened and endangered taxa has now been successfully propagated using *in vitro* techniques like micropropagation and somatic embryogenesis (Mandal *et al.,* 1995, Das *et al.,* 1997, Murthy and Saxena, 1998, Tiwari *et al.,* 1998, Cuenca *et al.,* 1999; Deb, 2001; Corredoira *et al.,* 2002, Deb and Tandon, 2002a, b, 2004a, b, Tyagi *et al.,* 2005, Sambyal *et al.,* 2006, Temjensangba and Deb, 2006; Deb and Temjensangba, 2006a, b, 2007a; Yarra *et al.,* 2010; Yapo *et al.,* 2011). *In vitro* mass propagation and reintroduction to the wild of selected rare plant will ensure continued presence of these plants in nature and use of plant/plant parts for commercial purpose. *In vitro* conservation of plant germplasm is increasingly being considered as the safer and more practical option compared to conventional approaches.

Cinnamomum tamala is a multipurpose plant used for medicines, spices, natural dyes etc. In view of the wide range of its uses, the species is harvested from the wild under different systems of regulations that vary with the states. This has put great pressure on the natural resources of the species. In addition, degradation in the natural habitat areas of the species owing to a variety of reasons, similar to many other forest tree species, has also contributed to the threats to the species. Some threats to the species were identified by Ved *et al.* (2003) in

Himalayan states of India. According to the report *C. tamala* has been found vulnerable in throughout the Himalayan region and North Eastern region of India, endangered in J&K. On the basis of identified threats and considering the economic potential and dwindling natural populations of *C. tamala* in several ranges, this species has been recommended for *in situ* as well as *ex situ* conservation (Samant *et al.* 2001). However, in the absence of standard agro-techniques and owing to lack of information on seed germination behaviors, conservation efforts have not succeeded so far. In view of these, the present study was undertaken on seed biology and development of culture systems for production of clonal planting materials under *in vitro* condition. ***In the present investigation for my Ph. D. programme, work has been done on the following aspects:***

1. **Study on seed biology like effect of developmental stages on *in vitro* morphogenetic response, effect of desiccation of seeds on seed germination both under *in vivo* as well as *in vitro* condition, post harvest storage of seeds at different temperatures.**
2. **Initiation of *in vitro* culture from different explants like embryos of various developmental stages, nodal explants, cotyledonary segments, leaf etc.**
3. **Optimization of physico-chemical factors for *in vitro* culture initiation.**
4. **Production of clonal planting materials.**
5. **Introduction the regenerates in the poly-house and natural habitat.**

Chapter - 2

Studies on Seed Biology of *Cinnamomum tamala*

A number of different plant species, both of tropical and temperate origin, produce seeds considered as recalcitrant because, differently from orthodox seeds, they are shed from the mother plant with high moisture content and are desiccation-sensitive. They generally directly pass from development to germination, even if in some cases a dormant phase occurs (Chin *et al.*, 1984; Berjak and Pammenter, 1994; Tommasi *et al.*, 2006). There are many types of recalcitrant seeds with different desiccation tolerance; moreover some species produce seeds with a behaviors intermediate between orthodox and recalcitrant (Berjak and Pammenter, 1994). For few recalcitrant seeds, there is consistent literature on some aspects of seed development, on the basic physiology and response to desiccation as well as ecology (Farrant *et al.*, 1993; Finch-Savage *et al.*, 1996; Pammenter and Berjak, 1999; 2000; Daw *et al.*, 2004; Pritchard *et al.*,

2004). However many questions are still open, for example concerning the life-span of seeds, long-term storage, temperature during storage, developmental stage of the seeds at which they should be harvested (Pammenter *et al.*, 1994), and also because there are a wide range of differences in the post harvest responses of recalcitrant seeds. Some data report that many recalcitrant seeds, particularly those of tropical origins, are also chilling sensitive and cannot be stored at temperatures below 15^{o}C. The storage life span is quite short varying from two weeks to some months (Chin and Roberts, 1980; Pammenter and Berjak, 1999).

Recruitment of tree species on the forest floor/natural habitat is governed by various factors including the seed traits, developmental stages of the embryos, desiccation tolerance of the embryos, microhabitat (Kitajima, 2007). Likewise, germination of seeds in nature as well as under *in vitro* condition is strongly influenced by internal as well as external environmental factors. The germinating seeds/embryos and seedlings are most vulnerable to predation, desiccation, competition and damage as the seeds and seedlings constitute important resource for the herbivores and pathogens. Disturbance in the form of trampling and habitat alternation, change in internal environment of the seeds have direct implications for recruitment and multiplication of the species. Consistency in microhabitat condition enhances seedling survival. One of the important microhabitat factors in the seed bed is light regime which is governed by the canopy cover in the forest. But in the artificial seed bed it may be compensated by appropriate cover of the seed bed. Tree species vary considerably in terms of light requirement at seedling stage (Davies, 2001). Germinating seeds, depending upon their state such as inherent properties, internal moisture content, reserve food, developmental stage at which they may be germinated optimally which have been rarely been investigated.

In terms of their ability to be stored seed-bearing plants fall into two categories, those that produce what are termed '**orthodox**' desiccation-tolerant seeds and those that have '**recalcitrant'**, desiccation-sensitive seeds. The ability of orthodox seeds to undergo maturation drying and withstand lowered temperatures has enabled humans to store this type of seed and, intact seed storage can be considered as ancient as agriculture itself. Orthodox seeds include most grain and legume types. Recalcitrant seeds, unlike their orthodox counterparts, do not undergo a period of maturation drying during their development, and as a consequence, they are shed from the parent plant at high water content. Since these seeds are sensitive to desiccation and also to chilling, they cannot be stored under the conditions that facilitate storage of orthodox seeds. At present the only practical method of conservation of these seeds is by storage at ambient temperatures and a relative humidity allowing maintenance of the seed water content at that characterizing, or only a little below, the newly shed state. This approach which is termed '**wet storage'** is useful only in the short-term. Because their seeds are short lived, and in order to maintain desirable varieties of crop species that produce recalcitrant seeds they are propagated vegetative means.

Cinnamomum tamala commonly called as '*Tejpat*' is an evergreen monoecious species distributed in the lower Himalayan zone. It is an important species in the transitional evergreen broadleaf forest. Natural stands of *C. tamala* are mostly found in shady moist habitats. Leaves are aromatic and traded as spice (Anonymous, 2006) and also as a source of various Ayurvedic formulations (Sarin, 2008). It flowers during March-May and usually pollinated by small insects such as honey bees. The fruits are ellipsoidal drupe. Ripe fruits are dark purple in color and contain single seed. The seeds are primarily dispersed by frugivorous birds, which feed on them

for nutritious pulp and egest the seeds intact. In addition, strong winds, hail storms and sometimes arboreal mammals such as primates may help in mechanical dispersal of fruits. Seeds are also secondarily dispersed by rodents and other small mammals (personal observation). Thus seeds of this species are deposited on the soil/forest floor in two states i.e., with or without pulp exhibiting different patterns of germination and establishment.

Considering the economic importance and dwindling natural populations of *C. tamala* in several ranges, the species demands *in situ* as well *ex situ* conservation. However, due lack of sufficient information on standard agro-technique, seed characters, seed germination behavior, conservation efforts have not succeeded completely. In view of the above, present study was aimed to study the role of various developmental stages of the embryo, desiccation tolerance of the seeds, temperature, effect of different light condition on seed storage, seed germination, seedling morphology both under seed bed condition and *in vitro* condition.

Materials and Methods

Site description: The study was conducted in the Department of Botany, Nagaland University, Lumami located in Zunheboto district of Nagaland ($26^{o}12'37''$ N altitude and $95^{o}29'28''$ E longitude; altitude 1150-1200 m asl). For the present study three different types of seed beds were prepared viz. 1. Seed bed with normal day light, 2. Seed bed in the poly shade with 50% filtered light and 3. Seed bed in the poly shade with 75% filtered light. All the seed beds were prepared by mixing humus (decomposed forest litter), soil, sand, decayed wood powder at 1:4:1:1 ratio respectively. The mixture was sundried before putting them in the seed bed (poly-bags).

Seed collection and transport: The details of collection and transport of different types of seeds for experimental purpose may materially affect result. For the present study, the intact mature fruits of about 16 weeks old were harvested randomly from the trees from two trees available in Mokokchung town, Nagaland during 2007-2009. The seeds were also collected from the ground near the selected trees which were freshly shaded. The collection was completely randomized without seeing the size and color of the fruits except the damage by the insects, microorganisms or birds. The collected seeds were transported to the Laboratory within 24 hours of collection in the plastic bags. In each fruit there was only one seed. On receipt, seeds were removed from the fruits, cleansed by rubbing with soft cloth towels, briefly surface cleansed with soft Laboratory detergent (1%, v/v, Extron a commercially available detergent, make: Merck, India) and stored in plastic bags at 25^{o}C. The time leg between removal of the seeds from the fruits and initiating experiments was 1-2 days. The seeds were made into different sub-lots/groups for different experiments. In each lot there were 50 seeds.

***Experimental process*:** The processed seeds were sowed immediately after harvest in the seed bed while rests of the seeds were treated differentially as described below:

1. A set of freshly processed seeds were sowed in three light conditions viz. normal light (5000 lux), poly house with 50% shade (2500 lux) and poly house with 75% shade (1250 lux).

2. To test the desiccation tolerance to the embryos/seeds, part of the seeds were desiccated/ dehydrated at various levels (100 to 30%, fresh weight basis). The rapid drying of the

seeds was achieved by exposing to cool blowing air and slow drying was achieved by exposing to the normal sunlight before they are sowed to the seed beds.

3. After processing one sub lot of seeds were stored without desiccating for various periods (0-18 days) at 4^{o}C (in the refrigerator) and 25^{o}C (in the Laboratory) in sealed poly-bags before they were sowed in the seed beds.

To study the emergence, survival and growth of seedlings of *C. tamala* under each condition, three replicates of 50 seeds each were used. In each experimental condition 50 poly-bags (15 cm long and 7.5 cm diameter) were used. In each poly-bag the soil mixture was packed. In each poly bag only one seed was sowed. The seed beds were watered weekly. The experimental design was completely randomized. The data was collected daily basis for seed germination, seedling morphology, per cent response etc.

The seedlings were maintained in the respective poly-bag and watered at regular interval and studied the seedling morphology for six months. Once the seedling showed normal functioning like formation of normal leaves, seedling growth etc., the seedling were transferred to the nature.

Results

Seeds sowed in the experimental seed beds started germinating and emergence of radicals within 10 days. The seed germination rate, germination time, morphology of seedlings was greatly influenced by illumination in the seed beds, level of desiccations, temperature of post harvest storage and duration. There was a significant variation in time taken for initial response

(10 to 15 days) and germination rate/seedling emergence (52.2 to 72.2%) across the seed beds with different light intensities (**Fig. 3**). Seeds sowed in the seed bed under higher illumination (5000 lux) under performed as there was delay in initial time taken for germination and overall germination rate (15 days and ~52% respectively). Compared to normal irradiation, seeds sowed under lower irradiations performed better. Of the different light conditions studied in the present study, optimum response was achieved from seeds sowed under 50% shade (2500 lux) where within 10 days of sowing radical started emerging and registered 72.2% seed germination (**Fig. 3**). Seeds sowed under normal light condition delayed germination and leaves were curly and exhibited stunted growth of the seedlings, while, seeds sowed under 75% shaded condition, seedlings were healthy but comparatively etiolated in comparison to 50% shaded condition.

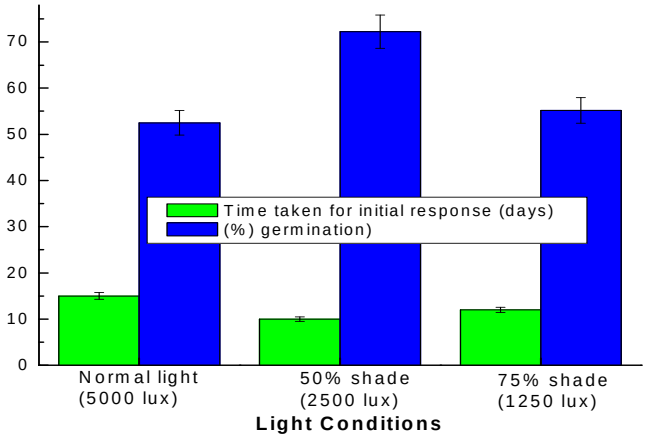

Figure 3: **Effect of different microclimatic conditions (light intensities) on seed germination of *Cinnamomum tamala* in the seed bed.**

Besides light requirement for seed germination, seeds were also tested for their desiccation tolerance, post harvest storage and temperature tolerance during storage. In the present study with *C. tamala*, the seeds sowed immediately after harvest exhibited highest

germination frequency (72.2%) after 10 days of sowing (**Table 1)**. The germination rate decreased considerably as the storage period increased exhibited <50% germination after 6th day of storage and after 16th day no seed germinated. As the storage period increased, germination period also delayed significantly. The seeds were stored in two different temperatures (4^{o}C and 25^{o}C). There was no significant difference in germination period as well as germination rate when compared in both the temperatures though experiments conducted at 4^{o}C performed showed slightly better (**Table 2**).

Table 1: Effect of desiccation of seeds on seed viability and seed germination of *Cinnamomum tamala* in the seed bed.

Desiccation level (%) (fresh weight)	**Time for first sign germination (days)**	**% germination (±SE)***	**Type of response**
100	10	72.2 (±1.5)[a]	Healthy seedlings with healthy roots
90	16	70.5 (±2.0)[a]	As above
80	19	71.5 (±1.5)[a]	As above
70	25	58.3 (±1.0)[b]	Delayed germination and seedling were stunted in growth
60	32	42.5 (±1.5)[c]	Dwarf seedlings with shorter leaves and poor rooting
50	35	35.2 (±1.5)[d]	As above
40	37	12.0 (±2.0)[e]	Seedlings with shorter leaves and roots but degenerated subsequently
30	-	-	No germination

* Standard error from mean.

Data represents the mean of three replicates.

In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

Table 2: Effect of post harvest storage of seeds at 4°C and 25°C on seed viability and germination in the seed bed.

Storage period (days)	Time taken for germination (days) from seeds stored at		% germination (±SE)* from seeds stored at	
	4°C	25°C	4°C	25°C
0	10	10	72.2 (±1.5)[a]	72.2 (±1.5)[a]
2	10	14	71.5 (±1.5)[a]	69.5 (±1.0)[a]
4	15	16	65.2 (±2.0)[b]	55.0 (±1.5)[b]
6	15	17	50.5 (±1.0)[c]	51.3 (±2.0)[b]
8	18	21	35.5 (±1.0)[d]	33.5 (±1.5)[c]
10	30	35	22.2 (±2.0)[e]	21.5 (±1.5)[d]
12	30	36	10.5 (±1.5)[f]	10.0 (±1.0)[e]
14	45	50	07.5 (±2.5)[g]	04.5 (±1.5)[f]
16	47	50	5.0 (±0.5)[g]	03.0 (±0.5)[f]
18	-	-	-	-

* Standard error from mean; Data represents the mean of three replicates.

In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

In the present investigation *C. tamala* seeds exhibited desiccation intolerance. The seeds sowed immediately after harvest without desiccation registered highest germination within minimal period of time. With desiccation, the viability decreased and registered poorer germination rate (**Table 2**). It was found that when the moisture content reduced to <60% (fresh weight basis) the germination rate decreased below 35% and abnormal seedling morphology was recorded which ultimately affected the seedling establishment in the seed beds as well as in the

nature. The seedlings developed from the seeds desiccated up to 70% exhibited normal seedling morphology and normal growth in nature (**Fig. 4a**). The seeds were desiccated following two methods i.e., rapid desiccation and slow desiccation. It was found that the seeds desiccation achieved by rapid method performed better in comparison to slow method. The seedlings formed from the seeds desiccated by rapid method were healthier and seedlings established better in the seed bed as well as in the nature (**Fig. 4b)**.

Figure 4: **a. Healthy seedlings in poly-bags germinated from the seeds, b. An established seedling converted into young plant in the field.**

Discussion

The practice of seed preservation is as old as agricultural practices but systematic collections and storage facilities have been a development of the 20th century. Presently there is an estimate over 1500 seed or gene banks around the world containing over 6 million seed accessions. For some plant species, using relatively fresh seeds gives superior germination over stored seeds. Viability of a seed lot declines over time and though old seed may germinate, the

resulting seedlings may have reduced vigor and fail to establish as well as seedlings from fresh seed (Walters, 2004).

The time for emergence of radicals from the germinated seeds, germination rate, seedling morphology and seedling establishment is influenced by various many factors. Plant species differ greatly in their habit preference, temperature requirement, post harvest storage, specific pre-treatment for seed germination, seedling emergence and survival. A number of tree species exhibit positive as well as negative correlation between canopy cover/light requirements, temperature etc. (Kwit and Platt, 2003; Pages *et al.*, 2003). The requirement of light for successful seed germination and healthy seedling morphology appears to be species specific. Seedling survival on the seed beds/forest floor is governed by the availability of light, water and nutrients (Kitajima, 2007). Increase in light and nutrients in mesic gaps stimulate the growth of the competing shrubs and fast growing herbaceous plants. In general, the varying microenvironment had a significant effect on survival of seedlings of French Alps (Pages *et al.*, 2003). According to Onen (1999), germination percentage was almost independent from light conditions. In mugwort the germination values obtained from light conditions were similar at the same incubation temperatures. Therefore, it was supposed that presence or absence of light had no effect on both germination percentage and germination speed of mugwort seeds. But, the fresh achenes germinated in room temperature under daily light were 14 per cent higher than that of under the dark condition (Onen, 1999). However, a brief exposure to low intense light was found sufficient to stimulate germination. But as the seeds aged, they became less dependent on light and eventually could germinate also in darkness (Holm *et. al.*, 1986). In the present study with *Cinnamomum tamala,* there was moderate germination in seeds sowed across the different

light conditions. In higher illumination the germination was slightly delayed compare to lower light intensity. From the other experiments, it was found that the seeds of *C. tamala* are recalcitrant in nature and does not go for dormancy. Present result is in agreement with the previous reports which describes that the non-dormant seeds of many species germinate well more or less equally in light and dark (Baskin and Baskin, 1988).

During the present study, investigations were carried out on the relationships among rate of drying, dehydration tolerance, post harvest storage and storage temperatures. From the results obtained in the present study clearly shows that *C. tamala* seeds are desiccation-sensitive. The seed material dried at the two rates used in this study showed considerable difference in seedling morphology and seedling establishment. The seedlings developed from the seeds desiccated by rapid desiccation method were healthy. The present observation is in agreement with Pammenter *et al*. (1991) where they have reported a similar response while working with *Landolphia kirkii*.

To determine seed type the seeds should be tested prior to storage. Seeds that tolerate desiccation to 5% moisture content or below (10-15% fresh weight of seeds) are likely to show orthodox seed-storage behavior. Seeds that tolerate desiccation to 10-12% moisture content (40-50% of fresh weight), but whose viability is reduced when subjected to further desiccation to a lower moisture content are likely to show intermediate seed storage behavior. Seeds that are killed by desiccation to 15-20% moisture content ~ 70% fresh weights of the seeds) are likely to be recalcitrant (Rao *et al*., 2006). In *C. tamala* dehydration of seeds below 60% reduced the viability of the seeds and germination. In addition to drying, another form of controlling the biological activities is the use of low temperature. Storing the seeds at 4^{o}C exhibited slightly higher percentage of viability and germination in comparison to storage at 25^{o}C. The lowest

temperature withstood by the recalcitrant seeds seems to vary with the species (Fu *et. al.*, 1990; Oliveira and Valio, 1992; Barbedo and Cicero, 2000; Tommasi *et al*., 2006). *Ginkgo biloba* seeds could be stored at 4^{o}C for one year but when stored at 25^{o}C, seeds died after 6 months (Tommasi *et al*., 2006). Present study with *C. tamala*, also exhibited a similar response where seeds stored at 4^{o}C germinated better over seeds stored at 25^{o}C.

The findings in the present study reveals that the seeds of *C. tamala* are desiccation-sensitive i.e., they are recalcitrant in nature and the seeds cannot be stored over a period of one week either at room temperature or at low temperature (4^{o}C). No differences were observed among germination rates of seed lots stored at different temperatures. Besides these, the seeds of this economically important species exhibit optimal germination and seedling establishment under diffused light condition. Further studies on mechanism of seed dispersal, seed storage and temperature tolerance will help in developing the conservation strategies of this economically important spice yielding species.

Chapter - 3

In Vitro Propagation of *Cinnamomum tamala*

Cinnamomum tamala is an economically important plant in India and mainly distributed in North-Western Himalayas, Sikkim, Assam, Mizoram, Meghalaya, Tripura and adjoining places. The leaves of *C. tamala* (*Tejpat*) are widely used as a spice and also yield an essential oil on distillation. The essential oil of the leaves called '*tejpat oil*' is medicinally used as carminative, anti-flatulent, diuretic, and in cardiac disorders (Showkat *et al.*, 2004). Parts of *C. tamala* are also used in many *Ayurvedic* preparations e.g. *sudarshan choorna* and *chandraprabhavati*. The leaf extracts are used as clarifiers in dyeing procedures with myrobalans. Traditionally green dye has been extracted from its leaves (Gaur, 2008). Besides its high economic importance, this species provides excellent habitat for a large number of

frugivorous birds and small mammals, which facilitate its regeneration in turn (Sharma *et al.*, 2009).

Owing to its high medicinal value and being an important ingredient of the spices the demand of *C. tamala* is increasing day by day and the species is being exploited from its natural pockets. Therefore, there is a need to raise high quality individuals in large scale to fulfill the increasing demand on the one hand and help the conservation of the species on the other. Successful *in vitro* establishment of aseptic cultures of any plant species depends on various factors like developmental stages of the explants sources, type of the explants, nutrient media, quality and quantity of the organic carbon sources, plant growth regulators, microclimatic conditions in the laboratory etc.

Materials and Methods

Plant collection

The present study was aimed for establishment of culture systems for production of cloning planting materials under *in vitro* condition and propagation of *C. tamala*. To achieve the overall objectives of the present study and initiation of cultures, the experimental materials like seeds of various developmental stages, young foliar explants and nodal explants were collected at different time interval from the two plants available in Mokokchung town, Nagaland, which is about 22 KM away from the Department of Botany, Nagaland University. The experimental materials were collected and transported to the Laboratory within three hours with appropriate care as described below and processed before initiation of culture.

Selection of Explants and Sterilization

Plant materials

***Seed explants*:** The immature seeds of various developmental stages (4 to 16 week after flowering, WAF) were harvested randomly from the two plants as mentioned above. The mature fruits of about 16 weeks old were harvested randomly from the trees available in Mokokchung town, Nagaland during 2007-2009. After harvest the freshly harvested fruits were soaked in citric acid solution (100 mg L^{-1}) and brought to the Laboratory and processed further for culture initiation. The collection was completely randomized without seeing the size and color of the fruits except the damage by the insects, microorganisms or birds. On receipt in the Laboratory, seeds were removed from the mature fruits, cleansed by rubbing with soft cloth towels, briefly surface cleansed with soft Laboratory detergent (1%, v/v, Extron a commercially available detergent, make: Merck, India) and stored in plastic bags at 25^{o}C but the seed coats of the immature seeds were left untouched.

Desiccation of mature seeds: The mature seeds were desiccated at various levels (100-30%, fresh weight basis) by exposing the seeds to blowing cool air at 25^{o}C to test their desiccation tolerance and effect on *in vitro* morphogenetic response.

Cotyledonary segments: The collected seeds were sterilized and embryos were excised out for culture. The cotyledons from these seeds were collected separately and cultured separately on basal medium with different growth adjunct.

***Nodal explants*:** The newly flushed twigs/shoots were collected from the mature plants throughout the year at one month interval and primed suitably. Immediately after harvest, the twigs were transferred to different antioxidant solutions like water, citric acid solution (100 mg L^{-1}), ascorbic acid solution (100 mg L^{-1}) and polyvinyl pyrollidone (PVP) (100 mg L^{-1}) till they are brought to the Laboratory and sterilized appropriately. The leaves and scale etc. were removed from the twigs and nodal explants was sterilized suitably and soaked in sterilized distilled water till cultured on medium. The pre-soaked and primed nodal segments were used for initiation of culture.

Foliar explants: The young leaves (within 2-3 days of opening) were collected from the freshly flushed twigs, soaked in distilled water and brought to the Laboratory.

Sterilization of explants

In the Laboratory the well expanded leaves were removed before nodal segments were scrubbed with diluted 'Labolene' (a commercial laboratory detergent, 1:100 ratio, v/v) and soft brush and washed under running water for 10 min. The processed seeds, nodal explants were sterilized with aqueous solution of $HgCl_2$ (0.3%, w/v) while for 5 min while 0.1% $HgCl_2$ for 5 min for foliar explants and subsequently rinsed 4-5 times with sterilized distilled water. Finally, the explants were dipped in ethanol (70%, v/v) for ~30 sec and rinsed with sterilized distilled water. After sterilization, the explants were soaked in sterilized water till culture.

Tissue Culture Media

For initiation of cultures from different explants, full strength MS medium (Murashige and Skoog, 1962) was used. The basal medium was fortified with three different organic carbon

sources (viz., dextrose, glucose and sucrose) at different concentrations (0-4%, w/v). The basal medium was further fortified with different quality and quantity of PGRs like α-naphthalene acetic acid (NAA), benzyl adenine (BA), kinetin (Kn) (0-12 µM) either singly or in combination and agar (0.8%, w/v) was used as gelling agent. Three different antioxidant types like ascorbic acid, citric acid and PVP at different concentrations (0-200 mg L^{-1}) with an increment of 50 mg L^{-1} was incorporated in the initiation medium. For leaf culture, MS medium were fortified with sucrose (0-4%) and agar (0.8%, w/v), PVP (200 mg L^{-1}). The medium was further fortified with different levels of PGRs like NAA, BA and Kn (0-12 µM) either singly or in combination.

The *p*H of all the media was adjusted to 5.6 using 0.1N NaOH and 0.1N HCl and about 12 ml medium was dispensed in each borosilicate test tube (size: 25x150 mm) and plugged with non-absorbent cotton. The media were sterilized by autoclaving at 121^{o}C for 20 min at a pressure of 1.05 kg cm^{-2}.

Plant Tissue Culture

Initiation of Cultures

Immature embryos: The embryos of various developmental stages (4-16 WAF) were excised out from the sterilized seeds and cultured on medium containing various levels of MS inorganic salts solution (0, 1/4th, 1/2 , 3/4th and full strength) but full strength of organic adjuncts. The basal media were fortified with different organic carbon sources like dextrose, glucose and sucrose (0-4%, w/v), different antioxidants (ascorbic acid, citric acid and PVP, 0-200 mg L^{-1}) and different quality and quantity of PGRs like BA and Kn (0-12 µM) either singly or in combination and agar (0.8%, w/v) as gelling agent. In each test tube one embryo was cultured and for each treatment 20 embryos were maintained. To study the effect of the seed cotyledons on the morphogenetic response of the immature embryos of *C. tamala*, a set of the embryos was excised with the lower

part of the cotyledons attached with the embryos and cultured while, the another set of the embryos was dissected out without the cotyledons. All the cultures were maintained at 25±2^{o}C under cool white fluorescent light provided by white CFL bulbs at an intensity of 40 µmol m^{-2} s^{-1} and 12/12 hr (light/dark) phase.

Culture of desiccated mature embryos: The mature seeds were also collected from the plants and seeds were desiccated at different levels (100-30%, fresh weight basis) following the process described above in chapter two. The dehydrated seeds were sterilized suitably and cultured on optimum initiation medium developed from the experiments with immature embryos.

Cotyledonary segments: The cotyledons were dissected out from the mature seeds. A part of the cotyledons were cut into two equal halves while, another part of the cotyledons were cultured intact. For initiation of culture, the cotyledons/segments were cultured on MS medium fortified with sucrose (3%), PVP (100 mg L^{-1}) and different PGRs like BA, Kn and TDZ (0-12 µM, either singly or in combination). The cultures were maintained in the normal Laboratory conditions. For each treatment 20 cotyledons/segments were cultured and in each test tube one cotyledon/segment were cultured.

Nodal explants: Sterilized nodal segments from distilled water were taken out and wiped on a sterilized tissue paper and cultured on different strength of MS medium (0, 1/4th, 1/2, 3/4th and full strength inorganic salts and full organic adjuncts) fortified with different organic carbon sources (0-4%, w/v), different antioxidants (0-200 mg L^{-1}) and different quality and quantity of PGRs like NAA, Kn and BA (0-12 µM) either singly or in combination and agar (0.8%) as gelling agent. About 1.0 cm long sterilized nodal segments were cultured on initiation medium

and the cultures were maintained at 25±2^{o}C under cool white fluorescent light at an intensity of 40 µmol m^{-2} s^{-1} and 12/12 hr (light/dark) phase. A set of nodal segments were also cultured immediately after harvest and sterilization but without priming as described above.

***Leaf*:** The young leaves were collected from the freshly flushed shoots and sterilized suitably. The half of the leaves was cut into two halves while other half of the leaves were left uncut/intact. The whole leaf and leaf segments were cultured on MS medium enriched with sucrose (3%, w/v), PVP (100 mg L^{-1}) and BA, Kn and NAA (0-12 µM either singly or in combination). The cultures were initially maintained in the dark for 2 days followed by in the normal Laboratory light condition (at 40 µmol m^{-2} s^{-1} light intensity and 12/12 hr, light/dark photo phase).

Initiation of callus culture

Besides the embryos cultured on cytokinin enriched medium, embryos were also cultured on auxin enriched medium. The embryos were cultured on MS medium fortified with sucrose (3%), PVP (100 mg L^{-1}), 2,4-D and NAA (0-9 µM) either singly or in combination. The calli formed on the initiation medium were maintained on MS medium supplemented with sucrose (3%) and optimum auxin concentrations from the initiation medium and BA (2 µM) for further proliferation. The cultures were maintained in the normal Laboratory condition as described above.

The resultant calli developed from the leaf explants, embryos and nodal explants were separated from the parent explants and maintained for two more passages on respective optimum initiation culture conditions for further proliferation. After adequate culture proliferation the calli were transferred on MS medium fortified with sucrose (3%), PVP (100 mg L^{-1}) and BA (6 µM) for two passages.

Experimental Design

A completely randomized experimental design was performed. In all the experiments, for each treatment 20 explants were maintained and all the experiments were repeated thrice. The cultures were maintained at 25±2^{o}C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} intensities and 12/12 hr each (light/dark) photo period unless mentioned otherwise. All the cultures were sub-cultured at 4-5 wk interval. *In vitro* response was evaluated based on the percentage of explants responded and number of propagules formed in the culture after specific period of time (as stated in the table) and data was expressed as the mean of replicates ± standard error.

Culture Proliferation and Plantlets Regeneration

The shoot buds/micro-shoots developed from the cultured embryos, cotyledonary segments and nodal segments were maintained on optimum initiation medium for another two passages. The young plantlets and shoot buds were then transferred on different strengths of MS medium containing different concentrations of sucrose (0-4%, w/v) and different PGRs such as BA and Kn (0-9 µM) either singly or in combination. The micro-shoots were separated at every sub-culture and transferred on fresh regeneration medium. The calli resulted from different explants were maintained on auxin free MS medium enriched with sucrose (3%) and BA and Kn (0-9 µM) for shoot bud formation and plant regeneration.

Rooting of Micro Shoots

Though there was some roots formation in some regenerated shoots on regeneration medium, but roots were not fully developed and shorter in length. About 4-5 cm long plantlets/shoots with well expanded leaves from the regeneration medium were selected for

inducing rooting. The micro-shoots were maintained on MS medium containing sucrose (3%), and NAA (0-8 µM) and maintained in normal laboratory conditions.

Hardening of Regenerates

The well rooted plantlets were taken out from the rooting medium and transferred on medium containing half strength MS inorganic salts and full strength organic additives fortified with sucrose (2%, w/v) devoid of any PGRs and maintained in normal laboratory conditions for 6-7 wk.

Potting Mix and Transplantation of the Regenerates

The hardened plantlets were taken out from the culture vials and washed with luke warm water to remove any traces of agar. The hardened plantlets were then transplanted on to plastic pots containing a mixture of soil, sand, decayed wood powder at 1:1 ratio with a moss topping. The pots were covered with holed transparent polybags and watered at week interval for two months. The transplants were fed with $1/10^{th}$ MS salt solution once in a week for 3-4 wk and maintained in poly-shade with Ca.75% of shading sunlight. After two months the acclimatized transplants were finally transferred to the field under normal full day light condition**.**

Results

Initiation of culture

Zygotic embryos

The immature embryos were cultured on different strengths of MS medium containing different supplements. Developmental age of immature embryos, moisture content/desiccation level of the seeds/embryos, presence or absence of cotyledons attached to the embryos, strength of the nutrient medium composition, quality and quantity of organic carbon, quality and quantity

of PGRs were found to be crucial factors for successful culture initiation. Swelling of the embryos followed by the induction of meristematic loci was the first sign of germination/morphogenetic response.

Effect of developmental stage of the embryo: In the present study with *Cinnamomum tamala*, *in vitro* morphogenetic response from immature embryos largely depended on developmental stage of the embryos at which they were harvested and cultured on medium. During the present study the seeds were harvested from 4-16 WAF and used for initiation of culture. Embryos upto 6 WAF did not show any sign of morphogenetic response. Though the embryos of 7 WAF exhibited initiation of morphogenetic response by way of swelling of explants and invocation of meristematic loci, but failed to convert into shoot buds/micro-shoots development and cultures degenerated subsequently (**Table 3**). The healthy shoot buds formed from the embryos of 11 WAF and optimum response was registered from the embryos of 12 WAF where ~60% embryos registered morphogenetic response where multiple shoot buds developed. The morphogenetic response initiated within 10 days of culture (**Fig. 5a**). Morphogenetic response declined significantly beyond the embryo age of 12 WAF (**Table 3**). It was found that the morphogenetic of embryos significantly governed by the presence or absence of the cotyledon attached to the embryos. In the present study, it was found that the cotyledon attached with the embryos out performed in terms of morphogenetic response in comparison to the embryos without the cotyledon attached with them. The resultant shoot buds/micro-shoots from the cotyledon attached embryos were healthier as well as higher in number against its counterpart (**Fig. 5 b, c**).

Effects of antioxidants**:** The embryos cultured on antioxidant free media resulted in release of phenolics in the medium and tissues turned necrotic. Incorporation of antioxidant in the medium

improved the condition. Of the different quality and quantity of antioxidants used in the present study, PVP at a concentration of 100 mg L^{-1} found to be most suitable. Compare to PVP, other two antioxidants (ascorbic acid and citric acid) across the concentrations tested under performed as far as controlling of browning is concerned (**Table 4**). Though both ascorbic acid and citric acid could reduce the phenolic oxidation, but did not support healthy culture initiation.

Table 3: Effect of developmental age of immature embryos of *Cinnamomum tamala* on *in vitro* morphogenetic response.

Age of the embryo (WAF)	% response (±SE)*	Morphogenetic pathway**	Type of response#
4	-	-	No response
5	-	-	No response
6	-	-	No response
7	10 (±0.5)^{f}	Ca	Callus of embryo but failed to proliferate and degenerated
8	17.5 (±1.0)^{2}	Ca	As above
9	23.2 (±1.5)^{d}	Ca-Sb	Shoot buds developed from the callus
10	27.6 (±2.0)^{c}	Sb, Ca-Sb	In some cases shoot buds developed but in most of the cases callus developed
11	45.5 (±1.5)^{b}	Sb	Direct shoot buds developed but failed to convert into plantlets
12	***60.5 (±(1.5)^{a}***	***Sb, Pl***	***Shoot buds followed by plantlets formed***
13	46.5 (±2.0)^{b}	Sb, Pl	As above but poor plant health
14	45.5 (±1.5)^{b}	Pl	Single plantlet formed with single root
15	45.0 (±1.5)^{b}	Pl	Cultures degenerated subsequently
16	45.0 (±0.5)^{b}	Pl	As above

* Standard error from mean; ** Ca: Callus; Sb: Shoot buds; Pl: Plantlets; # On MS medium supplemented with sucrose (3%, w/v), PVP (100 mg L^{-1}) and BA (12 µM); Data represents the mean of three replicates.

In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

Table 4: Effect of quality and quantity of antioxidants on *in vitro* culture initiation from immature embryos of *Cinnamomum tamala*.

Antioxidant type & concentration mg L^{-1})	**Type of response***
0	Release of phenolics and browning of medium as explants, stunted growth of cultures
Ascorbic acid	
50	As above.
100	Moderate browning of medium, did not support healthy growth of culture.
150	As above.
200	Browning controlled, but stunted growth of culture.
Citric acid	
50	Moderate browning of medium, did not support healthy growth of culture.
100	As above.
150	No browning of medium and explants, but failed to support healthy growth of culture.
200	No browning of medium but tissue became necrotic.
PVP	
50	Supports health growth of culture but slight browning of medium.
100	***No browning of culture medium, the growth of shoot buds developed is healthy*.**
150	As above but growth stunted slightly.
200	No browning of medium but did not support healthy culture growth.

*** On MS medium supplemented with sucrose (3%, w/v) and BA (12 µM).*

Response compiled from the observations from three replicates of experiments.

Effects of basal media: First sign of germination was observed as swelling of the embryos within 10 days of the culture which followed by shoot buds formation after 35 days of culture. The embryos were cultured on various strengths of MS medium. Of the different levels of the

MS medium tested, full strength of MS medium was found to be superior over other strength (**Fig. 6)**. At lower strengths of the basal medium delayed the morphogenetic response as well as the per cent response. On full strength of MS medium 60.5% of the embryos responded positively.

Figure 5: Different stages involved on *in vitro* immature embryo culture of *C. tamala*. a. Initiation of morphogenetic response from the cultured embryo, b. Performance of cultured embryos not attached to cotyledons, c. Initiation of multiple shoot buds from cultured embryos attached with cotyledons, d. Multiple shoot buds developed from the cultured embryos (advanced stage).

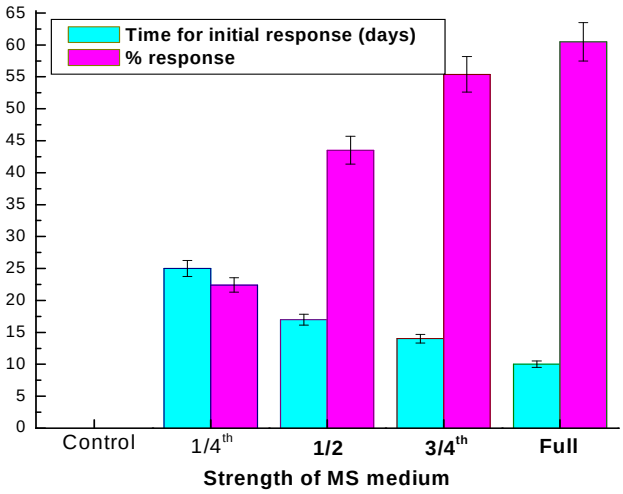

Figure 6: Effect of strengths of MS medium on *in vitro* culture initiation of *Cinnamomum tamala* using embryos as explants source.

Effects of different organic carbon sources: Different concentrations of various organic carbon sources viz., dextrose, glucose and sucrose (0-4%, w/v) were also incorporated in the initiation media. Incorporation of one of the organic carbon in the initiation medium was prerequisite for successful germination of embryos or induction of morphogenetic response. There was no morphogenetic response on organic carbon controlled medium. In general the morphogenetic response was very poor on medium fortified with dextrose as well as glucose as organic carbon source in across the concentrations tested. Amongst the different quality and quantity of organic carbons used, sucrose at a concentration of 3% supported optimum morphogenetic response and early differentiation (**Table 5**). At lower concentration of sucrose, fewer shoot buds formed while at higher concentration, the response was comparatively poorer.

Table 5: Effect of quality and quantity of organic carbon sources on *in vitro* culture of *Cinnamomum tamala* embryo.

Organic carbon source & concentration (%)	% response (±SE)*	Type of response**
0	-	No response
Dextrose		
1	5.2 (±0.5)^{g}	Only slight swelling of embryos but no shoot buds formed
2	14.5 (±2.0)^{f}	As above
3	36.6 (±1.5)^{d}	Only few shoot buds formed but not healthy
4	35.4 (±2.5)^{d}	As above
Glucose		
1	7.7 (±1.0)^{g}	Shoot buds etiolated
2	19.2 (±1.0)^{f}	As above
3	46.6 (±1.5)^{c}	Cultures were moderately healthy and fewer shoot buds formed
4	37.0 (±1.5)^{d}	As above and culture turned brown
Sucrose		
1	32.4 (±2.5)^{e}	Cultures were moderately healthy and fewer shoot buds formed
2	54.2 (±2.0)^{b}	Healthy shoot buds formed
3	***60.5 (±1.5)^{a}***	***Healthy and multiple shoot buds formed***
4	51.7 (±1.5)^{b}	Cultures were moderately healthy and fewer shoot buds formed

* Standard error from mean.
** On MS medium supplemented with PVP (100 mg L^{-1}) and BA (12 µM).
Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

Table 6: Effects of PGRs on *in vitro* morphogenetic response of cultured embryos of *Cinnamomum tamala*.

PGRs Conc. (µM) BA	Kn	% response	No. of loci (±SE)*	Type of response** developed
0	0	-	-	No response
3	-	52.5 (±1.0)^{c}	3.3 (±0.3)^{d}	Small shoot buds formed
6	-	56.2 (±1.7)^{b}	3.0 (±0.6)^{d}	As above
9	-	58.6 (±2.3)^{b}	4.6 (±1.0)^{c}	Healthy shoot buds formation
12	**-**	***60.5 (±1.5)^{a}***	***7.3 (±0.6)^{a}***	***Healthy shoot buds formation followed by plantlets formation***
-	3	32.1 (±0.6)^{f}	2.0 (±0.3)^{e}	Rooted shoots developed
-	6	57.5 (±1.5)^{b}	5.3 (±0.6)^{b}	As above but shoots were healthy
-	9	42.5 (±0.9)^{d}	3.3 (±0.3)^{d}	As above
-	12	18.2 (±1.0)^{h}	1.6 (±0.3)^{e}	Shoot bud not healthy
3	3	25.2 (±0.6)^{g}	3.3 (±0.3)^{d}	Healthy shoot buds
3	6	27.5 (±1.5)^{g}	3.0 (±0.7)^{d}	Shoot buds with root primordia
3	9	22.0 (±1.0)^{h}	2.0 (±0.6)^{e}	As above
3	12	-	-	Cultures turned brown
6	3	31.7 (±2.0)^{f}	3.6 (±0.3)^{d}	Shoot buds swelled at the base
6	6	37.0 (±2.0)^{e}	4.5 (±0.6)^{c}	As above
6	9	24.0 (±1.0)^{g}	2.0 (±0.6)^{e}	Swelling of propagule
6	12	-	-	No response
9	3	32.3 (±0.6)^{f}	2.3 (±0.5)^{e}	Shoots moderately healthy
9	6	30.0 (±1.0)^{f}	2.0 (±0.3)^{e}	As above but callusing at the base
9	9	30.0 (±1.0)^{f}	2.0 (±0.3)^{e}	As above but callusing at the base
9	12	-	-	No response
12	3	34.0 (±2.0)^{e}	2.6 (±.3)^{e}	Poor shoot growth
12	6	-	-	Callusing of the explants
12	9	-	-	No response
12	12	-	-	No response

Immature embryos of 12 WAF; * Standard error from mean; **On MS medium supplemented with sucrose (3%), PVP (100 mg L^{-1}); Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).

Table 7: Effect of desiccation of seeds on *in vitro* morphogenetic response of *Cinnamomum tamala* embryos[#].

Fresh weight of seeds (%)	Time for initial response (days)	% response (±SE)*	Type of response**
100	10	45.0 (±0.5)[a]	Healthy shoot buds developed with open leaf blade
90	12	45.00 (±2.0)[a]	As above
80	19	44.6 (±1.5)[a]	Few stunted shoot buds formed and leaves were curled
70	20	41.0 (±1.0)[b]	As above
60	25	32.0 (±2.0)[c]	As above
50	35	22.5 (±1.0)[d]	Only one shoot released but failed to produce leaves and degenerated
40	35	12.0 (±1.0)[e]	As above
30	-	-	No response, cultures degenerates

Embryos of 12 WAF; * Standard error from mean; ** On MS medium supplemented with sucrose (3%, w/v), PVP (100 mg L^{-1}) and BA (12 µM); Data represents the mean of three replicates; *In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error*).

Effects of quality and quantity of PGRs: For invocation of morphogenetic response from the cultured embryos, incorporation of PGRs was prerequisite. Amongst the different levels of PGRs used for culture initiation, Kn was found to be inferior over BA. Of the different quantities and combinations tested, singly treatment of both the PGRs was found to be superior over combined treatment. When tested singly, Kn at a concentration of 6 µM supported 57.5% response where as many as 5.3 shoot buds developed. But optimum response was registered on MS medium enriched with sucrose (3%) and BA (12 µM) where 60.5% explants registered morphogenetic

response and as many as 7.3 shoot buds developed per explants (**Table 6, Fig. 5d**). Amongst the combined treatments, optimal response was registered with BA and Kn (6 µM each) where 37% explants registered positive response and average 4.5 meristematic loci invoked per explants.

Effect of desiccation of seeds: During the present investigation on seed germination of *C. tamala* in the seed bed, it was found that mature seeds dehydrated below 60% (fresh weight basis), declined the germination significantly showing the recalcitrant nature of the *C. tamala* seeds. To test the same, the mature seeds were also desiccated at different levels and embryos were cultured on optimum initiation medium *in vitro*. There was no significant difference in morphogenetic response with reduction of moisture content of embryos up to 80% (fresh weight basis) (**Table 7**). Seeds desiccated below 50% reduced the response significantly and fresh weight of ~40% and below, morphogenetic response dropped to 12% and there was no *in vitro* morphogenetic response at fresh weight of 30%.

Cotyledonary segments

The cotyledons were excised out from the sterilized seeds, separated and cultured on MS medium fortified with sucrose (3%), PVP (100 mg L^{-1}) as antioxidant and different quality and quantity of PGRs. A part of the cotyledons were cut into two halves horizontally while the other half was cultured intact (**Fig. 7a**). In general, the intact cotyledons were found to be better over cotyledon segments. Within two week of culture, the responding cotyledons/segments started swelling and invoked meristematic loci (**Fig. 7b**). Amongst the three PGRs at different concentrations tested, BA across the concentrations found to be superior followed by TDZ and Kn respectively (**Table 8**). Under the given conditions, optimum response was registered on medium fortified with singly treatment of BA (6 µM). Under this condition 43.2% cotyledons

responded positively where as many as 6.6 shoot buds invoked per cotyledon (**Fig. 7b, c**). This was followed by 9 µM TDZ where 44.4% morphogenetic response was achieved. Incorporation of TDZ along with BA and Kn did not improved the morphogenetic response.

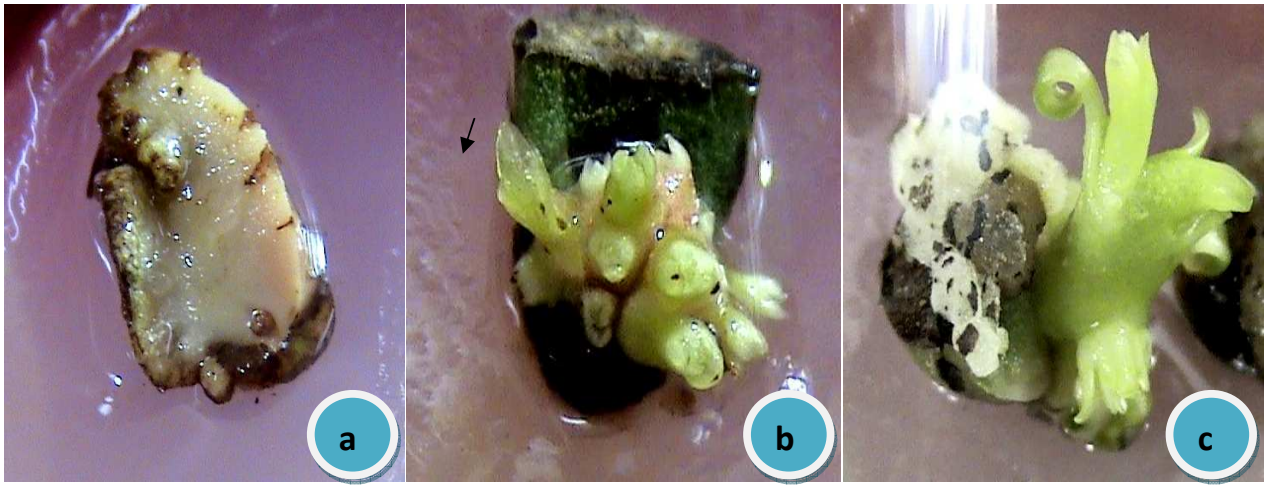

Figure 7: Different stages of *in vitro* morphogenetic response of *C. tamala* cotyledonary segments. a. A cultured cotyledon showing swelling, b. Multiple shoot bud formation from cultured cotyledon, c. Advanced stage/differentiated shoot buds developed from cotyledon. Nodal explants

The newly flushed shoots of *C. tamala* were collected from the mature trees throughout the year. The *in vitro* morphogenetic response was greatly influenced by various factors like post harvest treatments, seasonal effect of explants collection, strengths of basal medium, quality and quantity of organic carbon sources, PGRs, incorporation of antioxidants in the initiation medium.

Effect of post harvest priming of nodal segments: Immediately after collection of the newly flushed shoots, the nodal segments were soaked in different antioxidant solutions till used. The explants cultured from antioxidants control condition resulted into excessive browning of medium, tissue became necrotic and degenerated subsequently. Of the different antioxidant

solutions tested for pre-culture treatment, citric acid at a concentration of 100 mg L^{-1} reduced browning of medium and supported healthy culture initiation (**Table 9**).

Table 8: Effect of quality and quantity of PGRs on *in vitro* morphogenetic response of cotyledons of *C. tamala*.

PGRs Conc. (µM)			Morphogenetic	No. of loci	% response
BA	**Kn**	**TDZ**	**Pathway***	**invoked/explants****	**(±SE)*****
0	0	0	Sw	-	09.2 (±0.6)g
3	-	-	Sw-Sb	3.3 (±0.3)^{d}	22.5 (±1.5)^{e}
6	***-***	***-***	***Sb***	***6.6 (±0.6)^{a}***	***43.2 (±1.4)^{a}***
9	-	-	Sb	3.0 (±0.3)^{d}	16.6 (±1.2)^{f}
12	-	-	Sw-Ca-Sb	2.2 (±0.2)^{e}	14.2 (±0.7)^{f}
-	3	-	Sw-Sb	2.0 (±0.5)^{e}	38.0 (±1.7)^{c}
-	6	-	Sw-Sb	4.3 (±0.6)^{c}	40.6 (±1.3)^{b}
-	9	-	Sw	-	15.6 (±1.3)^{f}
-	12	-	-	-	-
-	-	3	Sw	-	16.2 (±0.7)^{f}
-	-	6	Sw-Sb	4.4 (±0.2)^{c}	33.3 (±1.6)^{d}
-	***-***	***9***	***Sw-Ca-Sb***	***5.6 (±0.4)^{b}***	***44.5 (±1.7)^{a}***
-	-	12	Sw-Sb	3.3 (±0.2)^{d}	22.2 (±1.2)^{e}
3	-	3	Sb	3.3 (±0.3)^{d}	34.2 (±1.0)^{d}
6	-	3	Sb	4.6 (±0.6)^{c}	41.0 (±1.5)^{b}
9	-	3	Sb	4.0 (±0.3)^{c}	36.6 (±1.2)^{c}
12	-	3	Sw-Sb	3.2 (±0.2)^{d}	26.3 (±1.6)^{e}
-	3	3	Sw-Sb	2.0 (±0.3)^{e}	23.2 (±1.8)^{e}
-	6	3	Sw	2.1 (±0.6)^{e}	22.0 (±1.5)^{e}
-	9	3	Sw	-	18.0 (±1.5)^{f}
-	12	3	Sw	-	20.2 (±1.2)^{e}

* Ca: Callus, Sb: Shoot buds, Sw: Swelling; **On MS medium enriched with sucrose (3%), PVP (100 mg L^{-1}); *** Standard error from mean; Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).

Table 9: Effect of post harvest priming of nodal explants of *Cinnamomum tamala* on *in vitro* morphogenetic response.

Type of pre-treatment	Type of response in culture*
Control	Excessive browning of medium, tissue became necrotic and degenerated subsequently.
Plain water	Reduced browning of medium, few nodal explants responded under optimum culture condition.
Citric acid (100 mg L^{-1})	***Reduced browning of medium, less tissue necrosis, shoot buds formed were healthy.***
Ascorbic acid (100 mg L^{-1})	As above but cultures are healthier.
PVP (100 mg L^{-1})	No browning of explants but did not support healthy culture growth.

Seasonal effect of explants collection: The first objective towards the establishment of *in vitro* regeneration protocol for *C. tamala* was to optimize the time of nodal explants collection from the field grown plants. After sterilization, the nodal explants were soaked in sterilized distilled water till culturing on nutrient medium. Soaking of nodal explants in water improved the morphogenetic response over non-soaked segments. It was observed that pre-soaked nodal segments leached lesser phenolics in the medium in comparison to non-soaked segments.

The nodal segments were collected round the year at one month interval starting from January till December. The explants were collected for three repeated years. In the present study with *C. tamala*, season of explants collection greatly influenced the explants establishment *in vitro* and successful initiation of culture. It was observed that amongst the different collection seasons, the nodal explants collected during October to April were least responding and tissues

turned necrotic. While, explants collected during June-August responded optimally where as much as 45.6% nodal explants responded by sprouting the axillary buds (**Table 10**). The explants collected September decreased the morphogenetic response significantly.

Table 10: Effect of season of collection of nodal explants of *Cinnamomum tamala* on *in vitro* morphogenetic response.

Month of collection	% response (±SE)*	Type of response**
January	-	No response, explants degenerated.
February	5.2 (±0.5)^{e}	Excessive browning of medium, only swelling of axillary buds.
March	5.1 (±0.5)^{e}	As above
April	8.5 (±0.6)^{e}	As above
May	22.7 (±0.9)^{c}	Moderate browning and few shoot buds sprouted from the axil.
June	44.5 (±1.0)^{a}	As above.
July	***45.6 (±1.5)^{a}***	***Less leaching of phenolics, healthy shoot buds formed.***
August	43.4 (±1.0)^{a}	As above.
September	35.4 (±1.5)^{b}	Excessive browning of medium and tissue became necrotic.
October	12.3 (±1.5)^{d}	As above.
November	-	No response, culture degenerated.
December	-	as above.

* Standard error from mean; **On MS medium supplemented with sucrose (3%), PVP (100 mg L^{-1}) and BA (9 µM)**;** Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).

***Effects of antioxidants*:** Under the conditions employed, the primed nodal segments were free from phenolic exudates; the resident axillary buds were induced to proliferate into shoot buds. On the antioxidant controlled medium the cultured nodal segments released excessive phenolics in the medium and tissue became necrotic. From necrotic explants morphogenetic response was

very poor and in most of the cases explants degenerated subsequently. For inducing morphogenetic response from the nodal explants, it was prerequisite to stop the exudation of the phenolic compounds in the medium. Various antioxidants viz. PVP, ascorbic acid and citric acid at various concentrations (0-200 mgL^{-1}) were incorporated in the initiation medium to stop/reduce the exudation of phenolic compounds (**Table 11**). Both ascorbic acid and citric acid across the concentrations tested though stopped the exudation effectively, but did not support healthy morphogenetic response. Under the conditions provided, PVP at a concentration of 100 mg L^{-1} reduced the leaching moderately and also supported healthy morphogenetic response.

Effects of basal media: Strength of basal medium played a vital role on morphogenetic response from nodal explants. At lower concentrations of MS medium delayed in the invocation of meristematic loci. With increase in strength of MS medium, morphogenetic response was also improved. Within 9 days of culture axillary buds started swelling on full strength of MS medium. About 46.5% explants exhibited morphogenetic response on full strength of MS medium (**Fig. 8**).

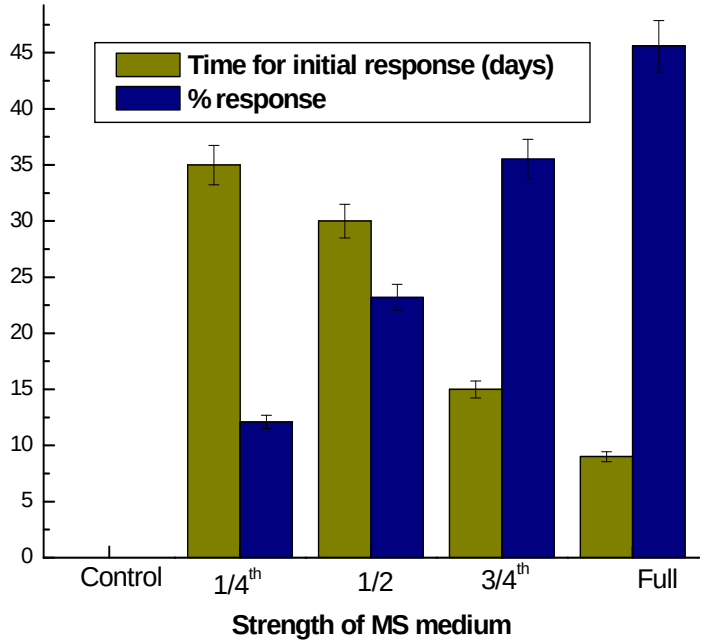

Figure 8: Effect of basal medium strength on *in vitro* culture initiation from nodal explants of *Cinnamomum tamala*.

Table 11: Effect of quality and quantity of antioxidants on *in vitro* culture initiation of *Cinnamomum tamala* nodal explants.

Antioxidant type & concentration mg L^{-1})	**Type of response***
0	Explants became dark brown and necrotic, release of phenolics and browning of medium as explants, explants degenerated.
Ascorbic acid	
50	Medium became dark due to phenolic exudation and cultures degenerated.
100	Moderate browning of medium, did not support healthy growth of culture.
150	As above.
200	Browning controlled, but stunted growth of culture.
Citric acid	
50	Moderate browning of medium, did not support morphogenetic response.
100	As above.
150	No browning of medium and explants, but failed to support healthy growth of culture.
200	No browning of medium but tissue became necrotic.
PVP	
50	Moderate morphogenetic response but slight browning of medium.
100	***No browning of culture medium, the growth of shoot buds developed is healthy*.**
150	As above but growth stunted slightly.
200	No browning of medium but did not support healthy culture growth.

** On MS medium supplemented with sucrose (3%, w/v) and BA (9 µM)**.**
Response compiled from the observations from three replicates of experiments.

Effects of organic carbon sources: Incorporation of at least one of the organic carbon sources in the initiation medium was obligatory. In the organic carbon control medium, there was no morphogenetic response and all the explants degenerated. In the present study, three different organic carbon sources (viz., dextrose, glucose and sucrose) at differential concentrations (0-4%) were incorporated. In general, amongst the three organic carbon sources used, glucose across the concentrations found to be inferior compared to other two sources. When glucose was incorporated as organic carbon, ~32% of the explants invoked meristematic loci on basal medium enriched with 3% glucose where as many as 4.5 shoot buds developed from each explant (**Table 12**). But when dextrose used as organic carbon, under optimum concentration

(4%) ~32% explants responded positively where average 3 numbers of shoot buds developed per nodal explants. Amongst the three carbon sources used in the initiation medium, sucrose at a concentration of 3% supported optimum morphogenetic response in 45.6% nodal segments after 6 wk of culture.

Table 12: Effect of different organic carbon sources on initiation of *in vitro* morphogenetic responses from nodal explants of *Cinnamomum tamala.**

Organic carbon source & concentration (%)	**% response (±SE)****	**No. of shoot primordial developed (±SE)****
0	-	-
Dextrose		
1	6.2 (±0.5)^{f}	1.3 (±0.33)^{e}
2	14.5 (±2.0)^{d}	1.7 (±0.33)^{e}
3	31.6 (±1.5)^{b}	3.0 (±0.20)^{c}
4	32.4 (±2.5)^{b}	3.0 (±0.33)^{c}
Glucose		
1	10.7 (±1.0)^{e}	1.7 (±0.33)^{e}
2	25.2 (±1.0)^{c}	3.0 (±0.33)^{c}
3	32.2 (±1.5)^{b}	4.5 (±0.33)^{b}
4	23.4 (±1.0)^{c}	3.2 (±0.60)^{c}
Sucrose		
1	23.5 (±2.5)^{c}	2.5 (±0.60)^{d}
2	32.5 (±2.0)^{b}	4.5 (±0.70)^{b}
3	**45.6 (±1.5)**^{a}	**5.4** (±0.40)^{a}
4	33.4 (±1.5)^{b}	5.0 (±(0.40)^{b}

* On MS medium supplemented with PVP (100 mg L^{-1}) and BA (9 µM)**.**

* Standard error from mean; Data represents the mean of three replicates.

In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

Effects of PGRs: Swelling of the axillary buds was observed within a week followed by differentiated into shoot buds/micro shoots formation within 5-6 wk (**Fig. 9a, b**). Presence of PGRs in the medium was necessary for morphogenetic response. In the absence of PGRs, there was only greening of the explants but degenerated without morphogenesis. Of the three PGRs incorporated at different concentrations, Kn in general found to be inferior over BA either singly or in combination with NAA. In the Kn enriched medium under optimum concentration (12 µM) only 36% explants responded positively where only 2 shoot buds formed per explants. When NAA (3 µM) was incorporated with Kn supported callus formation but when NAA was added with BA, supported shoot buds formation though response was not significant (**Table 13**). Under the given conditions the optimum response was registered on MS medium enriched with sucrose (3%), and BA (9 µM) where 45.6% nodal explants responded positively and formed 5.4 shoot buds per explants (**Fig. 9c**). When BA and Kn used in combination registered poorer response in comparison to singly treatment of BA.

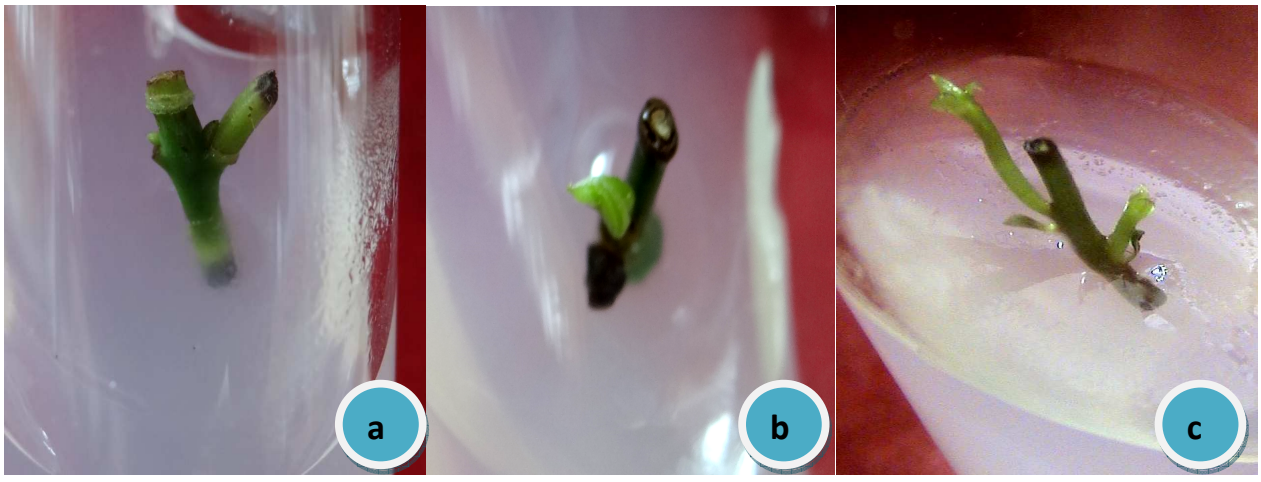

Figure 9: Stages involved on *in vitro* induction of morphogenetic response from the cultured nodal segments of *C. talmala*. a. Cultured nodal explants showing swelling of node and invocation of meristematic response, b. Nodal explants showing a shoot bud formation, c. Multiple shoot bud formation.

Table 13: Effects of PGRs on *in vitro* morphogenetic response from nodal explants of *Cinnamomum tamala.**

PGRs Conc. (µM)@			% response (±SE)**	Regeneration pathway***	No. of Shoot buds/loci formed
BA	Kn	NAA			
0	0	0	-	-	-
3	-	-	15.6 (±0.6)^{g}	Sb	1.0 (±0.3)^{d}
6	-	-	26.5 (±1.0)^{e}	Sb	2.3 (±0.3)^{c}
9	-	-	***45.6 (±1.5)^{a}***	***Sb-Pl***	***5.4 (±0.4)^{a}***
12	-	-	43.0 (±1.0)^{b}	Sb-Pl	4.6 (±0.6)^{b}
-	3	-	-	-	-
-	6	-	22.0 (±0.6)^{f}	Sb	1.0 (±0.3)^{d}
-	9	-	32.6 (±1.6)^{d}	Sb	2.0 (±0.3)^{c}
-	12	-	36.0 (±1.0)^{c}	Sb-Pl	2.0 (±0.3)^{c}
3	-	3	15.5 (±1.0)	Ca-Sb	1.0 (±0.15)
6	-	3	23.5 (±1.0)^{f}	Sb	2.3 (±0.3)^{c}
9	-	3	32.6 (±1.5)^{d}	Sb	2.6 (±0.6)^{c}
12	-	3	28.0 (±1.5)^{e}	Sb	2.0 (±0.3)^{c}
-	3	3	15.0 (±1.0)^{g}	Ca	-
-	6	3	31.6 (±1.5)^{d}	Ca	-
-	9	3	32.0 (±1.0)^{d}	Ca-Sb	1.6 (±0.3)^{d}
-	12	3	24.6 (±2.0)^{f}	Sb	1.3 (±0.3)^{d}
3	3	-	21.5 (±1.5)^{f}	Sb	1.6 (0.15)^{d}
3	6	-	17.0 (±1.0)^{g}	Sb	2.0 (±0.6)^{c}
3	9	-	15.0 (±1.0)^{g}	Sb	2.0 (±0.3)^{c}
3	12	-	-	-	-
6	3	-	22.0 (±1.5)^{f}	Sb	2.3 (±0.6)^{c}
6	6	-	27.6 (±1.5)^{e}	Sb	2.6 (±0.3)^{c}
6	9	-	15.6 (±1.3)^{g}	Sb	1.0 (0.0)^{d}
6	12	-	-	-	-
9	3	-	23.3 (±0.6)^{f}	Sb	2.3 (±0.6)^{c}
9	6	-	17.3 (±0.3)^{g}	Sb	1.3 (±0.3)^{d}
9	9	-	-	-	-

@ Only the significant treatments are computed; *On MS medium supplemented with sucrose (3%), PVP (100 mg L^{-1}); ** Standard error from mean; *** Ca: Callus, Sb- Shoot buds, Pl- Plantlet.
Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).

Table 14: Effect of PGRs on initiation of *in vitro* culture from foliar explants of *Cinnamomum tamala* from *in vivo* source.

PGRs Conc. (µM)			Morphogenetic pathway*	% response (±SE)**	Type of response
BA	Kn	NAA			
0	0	0	-	-	No response, explants degenerated
3	-	-	-	-	As above
6	-	-	-	-	Swelling of explants
9	-	-	-	-	As above
12	-	-	-	-	No response, explants degenerated
-	3	-	Ca	5.6 (±0.3)^{e}	Swelling of explants and poor callusing from the basal part of the leaf
-	6	-	Ca	12.0 (±0.6)^{d}	As above
-	9	-	-	-	No response
-	12	-	-	-	As above
3	-	3	Ca	25.6 (±1.0)^{b}	Initiation of callusing from the basal part of the leaf
6	***-***	***3***	***Ca***	***28.5 (±0.6)^{a}***	***Initiation of callusing throughout the leaf/segments***
9	-	3	Ca	23.0 (±1.0)^{b}	As above but comparatively harder
12	-	3	Ca	15.6 (±1.5)^{d}	As above
-	3	3	Ca	21.0 (±2.0)^{c}	Initiation of callusing in the mid vein of the leaf
-	6	3	Ca	23.2 (±1.5)^{b}	Callusing from the leaf base as well as from leaf vein
-	9	3	Ca	18.0 (±0.6)^{c}	As above but cultured turned brown
-	12	3	-	-	Cultures turned brown

*On MS medium supplemented with sucrose (3%), PVP (100 mg L^{-1}); ** Standard error from mean.
*** Ca: Callus; Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).

Foliar explants

Soaking of leaf explants in water after excising from the *in vivo* grown plants improved the morphogenetic response over non-soaked explants. It was observed that pre-soaked leaf

explants leached phenolic in the water and promoted healthy culture initiation. The foliar explants/segments were cultured on MS medium fortified with sucrose (3%), PVP (100 mg L^{-1}) and three different PGRs (BA, Kn and NAA) either singly or in combination. In general when used singly BA across the concentrations failed to invoke any response and Kn enriched medium supported partial swelling of the explants. But when either of the cytokinins was conjunct with NAA supported callus formation (**Table 14**). A part of the leaves was cut into two trans-segments and the other half was cultured intact. Of the two different types of explants cultured, better morphogenetic response was achieved from the intact leaves. Within 3 days of culture the responding leaves curled and callus formation was started from after 15 days of culture (**Fig. 10a)**. The callus formation was started from the basal part of the leaf which extended throughout the leaf (**Fig. 10b)**. In the present study with the leaf explants of *C. tamala*, ~28% leaf exhibited callus formation on basal medium fortified with BA (6 µM) and NAA (3 µM) in combination (**Table 14**).

Initiation of callus culture from embryos: The zygotic embryos cultured on auxin rich medium exhibited differently in comparison to cytokinin enriched medium. In the present study two different auxins (2,4-D and NAA) either singly or in combination was incorporated. All the treatments were further fortified with BA (2 µM). In general the embryos cultured on 2,4-D fortified medium under performed in comparison to NAA fortified medium when incorporated either singly or in combination. On 2,4-D fortified medium optimum response was recorded with 9 µM 2,4-D (**Table 15**) where 45.6% explants formed callus after 48 days of culture. Across the concentrations of combined treatment of 2,4-D and NAA did not support healthy callus initiation. In the present study optimum response was achieved on MS medium fortified with

sucrose (3%), PVP (100 mg L^{-1}), NAA (6 µM) and BA (2 µM) where within 35 days of culture 55.5% of cultured embryos converted into callus (**Table 15, Fig. 10c**).

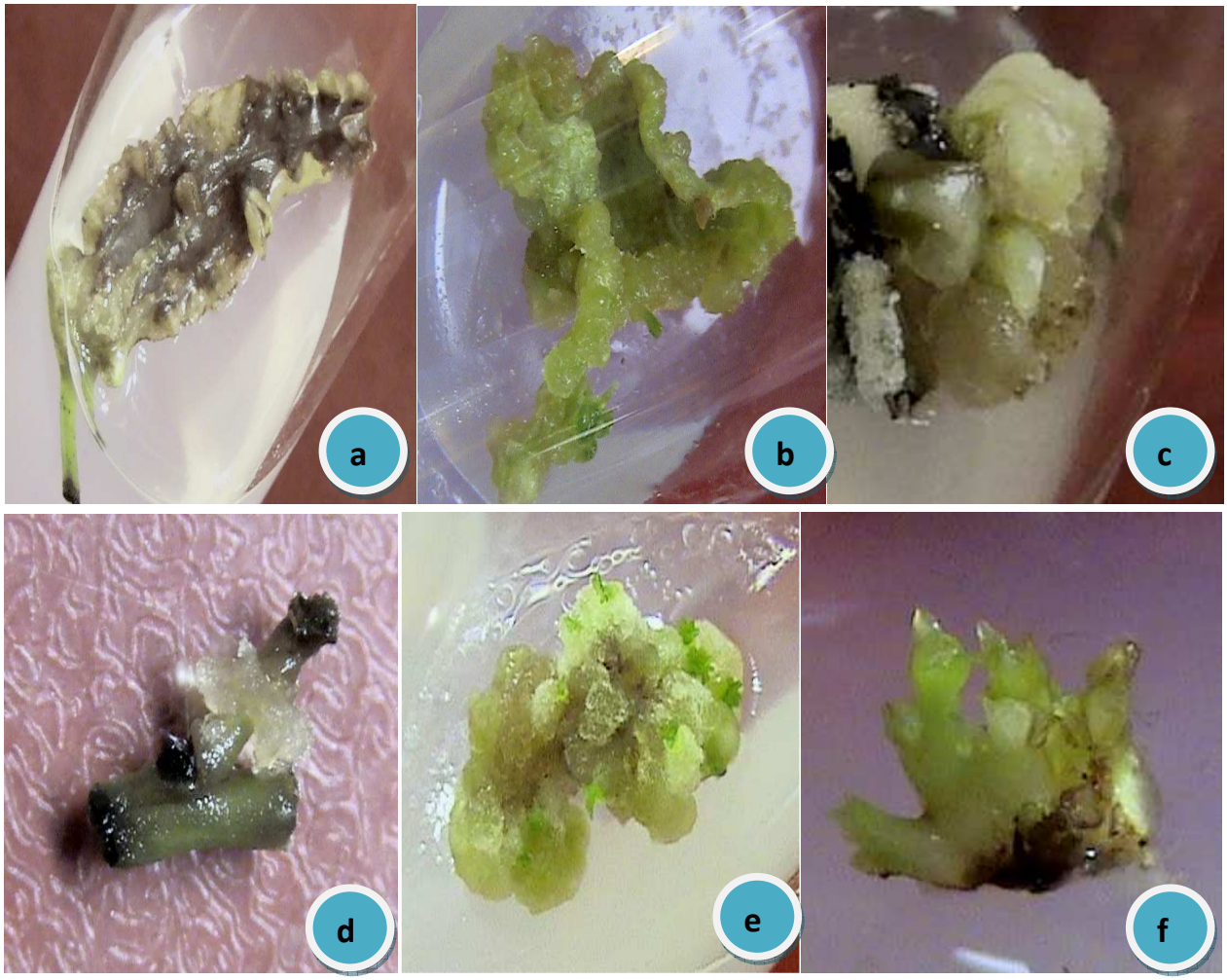

Figure 10: Callus induction from different explants of *C. tamala* and shoot bud formation. a. callus formed throughout the leaf surface, b. Callus proliferation and induction of shoot buds from leaf raised callus, c. Callus formed from cultured embryo, d. callus formed from cultured nodal explants, e. Callus proliferation and shoot bud induction and f. Shoot buds developed from the cultured callus.

The calli developed from the leaf explants, embryos and nodal explants (**Fig. 10d**) were separated from the parent explants and maintained for two more passages on optimum initiation condition for further proliferation (on MS medium fortified with 3% sucrose, 6 µM BA and 3

µM NAA). After adequate culture proliferation the calli were transferred on MS medium fortified with sucrose (3%), PVP (100 mg L^{-1}) and BA (6 µM) for two passages. On this condition shoot buds developed from the proliferated calli (**Fig. 10e, f**).

Table 15: Effect of quality and quantity of auxins on initiation of callus culture of *C. tamala* from immature embryos of 12 WAF.

PGRs Conc. (µM) 2,4-D	NAA	Morphogenetic pathway*	Time taken for response (days)**	% response (±SE)***
0	0	-	-	-
0	3	Sw-Ca	55	38.2 (±1.6)[c]
0	***6***	***Ca***	***35***	***55.5 (±2.5)[a]***
0	9	Ca	32	52.0 (±1.5)[a]
3	0	Ca	56	32.5 (±1.5)[d]
6	0	Ca	50	44.5 (±2.0)[b]
9	0	Ca	48	45.6 (±2.5)[b]
3	3	Sw-Ca	42	38.2 (±1.5)[c]
3	6	Ca	40	48.6 (±1.5)[b]
3	9	Ca	42	41.2 (±2.2)[c]
6	3	Sw-ca	45	39.2 (±3.6)[c]
6	6	Ca	40	41.6 (±1.7)[c]
6	9	Ca	40	41.5 (±1.2)[c]
9	3	Ca	49	37.6 (±1.2)[c]
9	6	Ca	45	32.3 (±0.7)[d]
9	9	Ca	45	30.7 (±1.3)[d]

* Ca: Callus, Sw: Swelling; **On MS medium enriched with sucrose (3%), PVP (100 mg L^{-1}) and BA (2 µM); *** Standard error from mean; Data represents the mean of three replicates.
In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).

Culture Proliferation and Plant Regeneration

The meristematic loci/shoot buds/micro-shoots developed from explants like embryos, cotyledons and nodal segments on initiation medium were maintained for another two passages for culture differentiation and proliferation on the respective initiation media and supplements. The micro-shoots/shoot buds were then maintained on different strengths of MS medium containing various level of sucrose as organic carbon source (0-4%) and two different cytokinins (BA and Kn) at various concentrations (0-9 µM singly or in combination) for plant regeneration and culture proliferation. The regeneration of plantlets and culture proliferations were influenced by various factors.

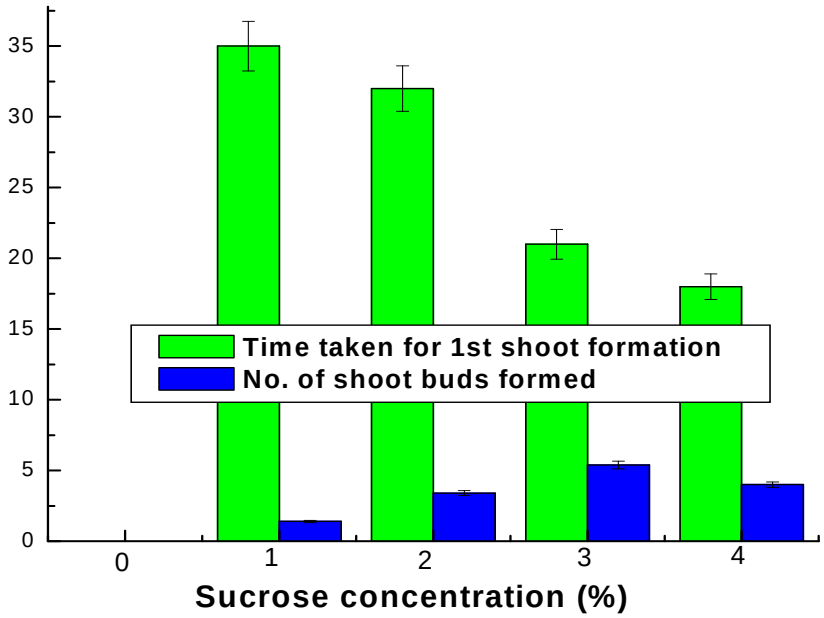

Figure 11: Effect of different concentrations of sucrose on regeneration of plantlets and culture proliferation of *Cinnamomum tamala*.

Effects of sucrose concentration: It was found that presence of sucrose carbon in the regeneration medium was prerequisite by the fact that in the absence of sucrose there was no regeneration of plants and cultures degenerated within 2-3 week after culture. Presence of sucrose at lower concentrations delayed the culture differentiation. At 1% sucrose differentiation started after 35 days of transfer on regeneration medium where ~1.4 shoots buds per explants developed. With increase in sucrose concentration, the culture differentiation and proliferation improved considerably (**Fig. 11**). Under the given conditions optimum response was registered on MS medium enriched with 3% sucrose, where healthy plantlet regeneration was recorded. At higher concentration the regenerative response declined.

Table 16: Effect of different strengths of MS medium on regeneration of plantlets and culture proliferation of *Cinnamomum tamala*.

Strength of medium	No of shoot buds formed per subculture (±SE)*	Type of response**
0	-	No regeneration, culture degenerated.
1/4th strength	1.0 (±0.3)	Single plantlet, no proliferation, poor growth of the plant.
1/2 strength	2.4 (±0.5)	Poor plant growth, leaves poorly developed.
3/4th strength	3.2 (±0.3)	Moderately healthy plantlets with well expanded leaves.
Full strength	5.4 (±0.4)	Healthy plantlets with newer shoot buds formation.

* Standard error from mean; **On MS medium supplemented with sucrose (2%), PVP (100 mg L^{-1}) and Kn (3 µM); Data represents the mean of three replicates.

Effect of strength of basal medium: The strength of nutrient medium influenced significantly the culture differentiation and plant regeneration. At lower strength of MS medium delayed the

response and shoot growth was poorer. With increase in basal medium strength improved the plant growth as well as culture proliferation. Optimum response was registered on full strength MS medium (**Table 16**).

Table 17: The role of different cytokinins on plant regeneration and culture proliferation of *Cimmamomum tamala*.

PGRs Conc. (µM)#		No. of shoot buds formed/explant	Mean plantlet height (cm.)*	Type of response**
BA	Kn			
0	0	0	0	Growth stunted and degenerated
3	0	3.2[b]	3.2±0.1[c]	Plantlets with dark green small leaves but plantlets etiolated
6	0	2.6[c]	4.0±0.1[b]	Plantlets slightly light green and not healthy
9	0	2.2[c]	3.6±0.2[b]	Small leaves and slight callusing at the base
0	***3***	***5.3[a]***	***4.5±0.2[a]***	***Healthy plantlets with dark green and broad leaves, plantlets with few roots***
0	6	3.2[b]	3.0±0.3[c]	Poor plant growth, yellowish-green leave
0	9	2.1[c]	2.9±0.2[c]	Stunted plant growth
3	3	3.2[b]	4.3±0.1[a]	Dark green leaves, stunted growth with fewer roots
3	6	3.3[b]	3.5±0.3[b]	As above
3	9	3.2[b]	3.6±0.2[b]	As above
6	3	3.4[b]	3.0±0.2[c]	Stunted plant growth and leaves light green
6	6	2.3[c]	2.7±0.2[d]	As above
6	9	3.2[b]	3.2±0.5[c]	Many small leaves crowded at top, leaves light green
9	3	2.5[c]	2.7±0.1[d]	Broad green leaves, healthy plantlets
9	6	3.2[b]	2.5±0.2[d]	As above
9	9	2.3[c]	2.9±0.2[c]	Leaves light green and plantlets unhealthy

Only the significant treatments are computed; * Standard error from mean; Data represents the mean of three replicates; Data scored after 8 wk of culture on regeneration medium; ** On MS medium, supplemented with sucrose (2%), PVP (100 mg L^{-1}); *In the same column, figures followed by the same letter were statistically identical to the threshold of 5% (Newman-Keuls, ±standard error*).

Table 18: NAA stimulated *in vitro* rooting of micro shoots of *Cinnamomum tamala*.

NAA Conc. (µM)	No. of roots formed/plantlet	No. of secondary shoot formed/shoot	Type of response*
0	3^{d}	-	Roots were very small and degenerated.
1	5^{c}	2	Plantlets etiolated, roots short.
2	8^{b}	3	Slightly etiolated plantlets, shoots branched but short
3	***10[a]***	***3***	***Healthy plants with profuse rooting with distinct root hairs***
4	9^{b}	2	Healthy roots but poor root hairs
5	6^{c}	2	Swelling at the basal part of plants as well as roots.
6	6^{c}	-	As above.
7	5^{c}	-	Roots swelled and callusing at the base of the shoot
8	4^{c}	-	As above

* On MS medium containing sucrose (3%, w/v); Data represents the mean of three replicates.
Data scored after five wk of culture on the above medium; *In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls).*

Effect of different cytokinins: For regeneration of plantlets, incorporation of one of the PGRs was obligatory. In medium freed of any growth regulators, cultures exhibited stunted growth and degenerated subsequently. Presence of BA across the concentrations either singly or in combination did not promote healthy plant regeneration or culture proliferation and plantlets exhibited stunted growth. Singly treatment of BA (3 µM) supported mean 3.2 numbers of shoot bud formation after 7 wk of culture where mean height of plantlets was 3.2 cm. While BA and Kn in combination exhibited a more or less a similar response in the entire range studied and did not support optimum plant regeneration and culture proliferation. Under optimum condition at a concentration of 6 µM BA and 3 µM Kn formed 3.4 shoot buds and mean plant height was 3.0

cm. But incorporation of Kn singly proved to be beneficial under the given conditions for shoot proliferation and plant growth. A maximum of 5.3 shoot buds/micro-shoots of average 4.5 cm height was achieved recorded on MS nutrient medium containing Kn (3 µM) singly (**Table 17, Fig. 12a**). The regenerated micro-shoots were maintained on the optimum regeneration medium for another two passages for further growth.

Rooting of Micro Shoots

Though there was roots formation in some regenerated micro shoots on regeneration medium, but roots were not fully developed and shorter in length. Regenerated micro shoots (~4-5 cm in length, sourced directly from regeneration medium) were transferred on medium for inducing roots in micro-shoots (**Fig. 12b)**. The micro shoots were treated differently for inducing roots. The micro-shoots were cultured on MS medium fortified with sucrose (3%) and NAA (0-8 µM). Of the different strengths of NAA tested, healthy plant growth as well as roots was achieved on medium supplemented with NAA (3 µM) where as many as 10 roots per micro shoot developed (**Table 18, Fig. 12c**) after 5-6 wk of culture. At higher concentration of NAA stimulated swelling as well as callusing of the shoots at the base.

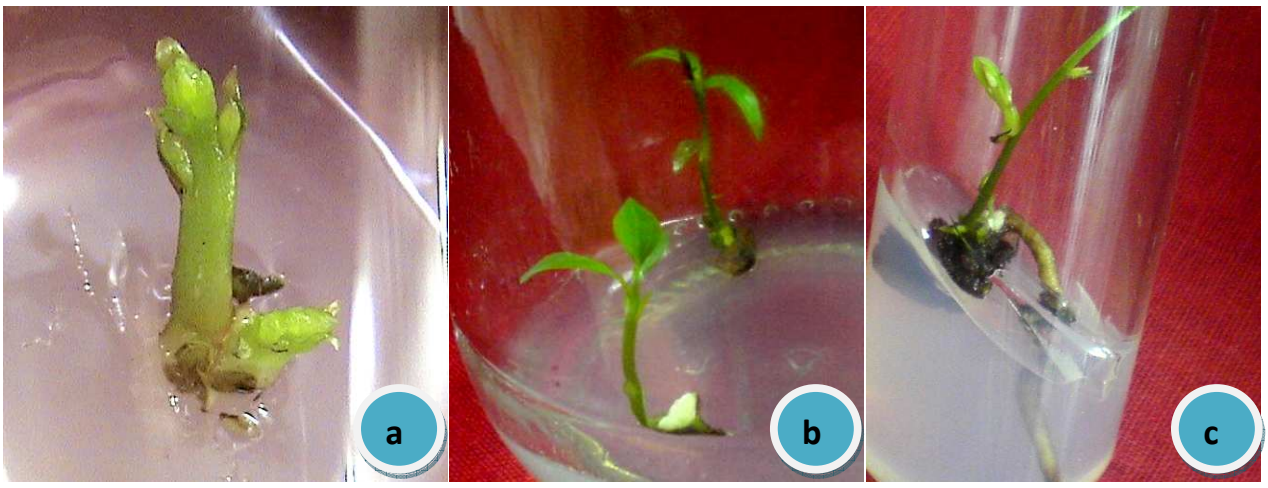

Figure 12: a. Shoots buds cultured on regeneration medium developed on initiation medium from different explants, b. Regenerated plants on regeneration medium, c. A plant showing formation of rooting.

Hardening of the Regenerates and Transplantation to CPM

The well rooted plants from rooting condition were transferred on medium with ½MS salt solution containing sucrose (2%) and freed from any PGRs and maintained for 6-7 wk under normal laboratory condition. The hardened plantlets were taken out from the culture vials and washed with luke warm water to remove any traces of agar. The hardened plantlets were then transplanted oncto plastic pots containing a mixture of soil, sand, decayed wood powder at 1:1 ratio with a moss topping (**Fig. 13a**). The pots were initially covered with holed transparent poly-bags for retaining moisture and watered at week interval for about two months. The plants were fed with 1/10th MS salt solution once in a week for 3-4 wk and maintained in poly-shade with Ca.75% of shading sunlight. Finally after two months of acclimatization the transplants were transferred to the natural habitat under normal sun light (**Fig. 13b).** About 200 plants were tested for survival and about 70% survival was registered after two months of transfer.

Figure 13: a. Rooted plants transferred to potting mix established in the poly house, b. A transplant established in the natural habitat.

Discussion

Initiation of culture

Zygotic embryos

The immature embryos of several commercially viable and or threatened taxa were used successfully for micropropagation and mass multiplication (Sharma and Tandon, 1990; Temjensangba and Deb, 2005a, c). The successful seed germination and or immature embryo culture are greatly influenced by several factors like developmental age of the embryos/seed, quality and quantity of nutrient media, different media supplements, quality and quantity of PGRs and other culture conditions (Deb and Sungkumlong, 2008, 2009; Deb and Temjensangba, 2006b, 2007b; Sharma and Tandon, 1990; Sungkumlong and Deb, 2008; Temjensangba and Deb 2005a, 2006, Pongener and Deb, 2009, 2011a).

Effects of developmental stage of embryos: A key factor for successful *in vitro* initiation of culture to a great extend depends on the right developmental stage of the embryos. Different species exhibit a particular threshold, a factor genetically structured in the organism. The influence of physiological age varies with the genus, species within the genus (Jamir *et al.*, 2002; Temjensangba and Deb, 2005a; Deb and Temjensangba, 2006a; Sungkumlong and Deb, 2008; Godo *et al.*, 2010; Pongener and Deb, 2011a). There is a window period of seed development for every species, which supports optimum *in vitro* morphogenetic response. The earliest stage at which the embryos could be cultured successfully varies within the genotype and local conditions (Sauleda, 1976; Temjensangba and Deb, 2005a, Deb and Temjensangba, 2006b; Pongener and Deb, 2009, 2011a). Therefore it is desirable to determine the right developmental stage of the seeds/fruits to harvest the green seeds/fruits for achievement of an optimal response.

The *Cinnamomum tamala* seed reaches maturity after ~16 WAF. In the present, seeds up to 6 WAF did not exhibit any response while, the cultured mature seeds did not respond optimally. In the present investigation, the seeds age of 12 WAF supported optimum morphogenetic response where ~60% of the cultured embryos responded positively and formed shoot buds (Table 3). The ability of immature embryos to respond better than the mature ones is due to their distended testa cells and metabolically awakened embryos and lack of dormancy and inhibitory factors (Yam and Weatherhead, 1988, Pongener and Deb, 2009, 2011a).

***Effects of strength of basal medium*:** In the present investigation, different strengths of MS medium were tested for culture initiation from the immature embryos of *C. tamala*. Of the different strengths of the MS medium tested, optimum morphogenetic response was registered on full strength of MS medium. Lower strengths of MS medium were found to be inferior in comparison to full strength of MS medium. The different species exhibit a preferential requirement to specific nutrient media for seed germination/morphogenetic response but as such no standard media can be prescribed for all the taxa. Deb and Temjensangba (2006a) reported better seed germination of *Malaxis khasiana* on MS medium while, *Arachnis labrosa* on Mitra *et al.* medium (Temjensangba and Deb, 2005a), *Cleisostoma racemiferum* on MS medium (Temjensangba and Deb, 2006), *Coelogyne suaveolens* on MS medium (Sungkumlong and Deb, 2008), *Cymbidium aloifolium* on MS medium (Pongener and Deb, 2011a), *Cymbidium macrorhizon* on Mitra *et al.* medium (Vij and Pathak, 1988), *Cymbidium iridioides* on MS medium (Pongener and Deb, 2009), *Dactylorhiza hatagirea* in Knudson 'C' medium (Vij *et. al.*, 1995), *Dendrobium chrysotoxum* in Vacin and Went medium (Rao *et al.*, 1998), *Dendrobium primulinum* on MS medium (Deb and Sungkumlong, 2009), *Eulophia alta* on '*Phyto*Technology

Orchid Seed Sowing Medium' (Johnson *et. al.*, 2007), *Geodorum densiflorum* on PM medium (Bhadra and Hossain, 2003).

Effects of antioxidants: In many plant species release of phenolic compounds from the cultured explants in the medium followed by browning of medium and tissue necrosis is a common problem. In the culture of *C. tamala,* the exudation of phenols and browning of medium could be prevented by incorporating antioxidant like PVP (100 mg L^{-1}). Deb and Tandon (2004b) could successfully control the browning of medium by incorporating 200 mg L^{-1} PVP and 100 mg L^{-1} citric acid in combination in *Pinus kesiya.*In the present study with the immature embryos of *C. tamala* incorporation of citric acid and ascorbic acid did not improve the culture condition.

Effects of organic carbon sources: Besides the other factors, quality and quantity of organic carbon sources present in the culture medium exerted pronounced effect on morphogenetic response of *C. tamala* immature embryos. The carbon sources serves as energy and osmotic agents to support the growth of plant tissues. The process of *in vitro* culture establishment is a highly energy requiring processes that can occur at the expense of available metabolic substrates like organic carbon sources. In the present study, for culture initiation from immature embryos, three different organic carbon sources at differential concentrations (viz., dextrose, glucose and sucrose at 0-4%) were incorporated in the medium. In absence of organic carbon source, all explants degenerated. Optimum response was recorded on medium containing sucrose (3%) while other carbon sources did not support optimal culture initiation. Earlier, the effect of organic carbon source on *in vitro* morphogenetic response was described in *Stevia rebaudiana* (Preethi *et al.*, 2011). The requirements of the quality and quantity of exogenous supply of the organic carbon sources vary with the species, the media compositions used, the endogenous level

of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005c, Pongener and Deb, 2009, 2011a).

***Effect of PGRs*:** In the present investigation, for invocation of morphogenetic response from the cultured embryos, incorporation of PGRs was perquisite. In the absence of PGRs there was no response and all the explants degenerated. The PGRs in the initiation medium showed a marked effect on the growth, differentiation. Kinetin either singly or in combination with BA across the concentrations found to be inferior. Optimum morphogenetic response and healthy culture growth was registered on MS medium fortified with sucrose (3%) and BA (12 µM). The present observation is in contradiction with Sharma and Tandon (1986); Li and Xu (2009) where they reported the stimulatory effect of NAA in conjunction with cytokinins. In *Rhynchostylis gigantea* NAA (0.2 mg L^{-1}) and BA (0.05 mg L^{-1}) supported optimum germination. But Nagaraju *et al.* (2004) reported that in *Dendrobium* hybrid, the single leaflet in the PLB developed when basal medium was supplemented by BAP alone while, BA singly supported better germination in *Malaxis khasiana* (Deb and Temjensangba, 2006a).

Effect of desiccation of embryos on morphogenetic response: Recruitment of tree species in the forest is governed by various factors including developmental stages of the embryos, desiccation tolerance of the embryos. Similarly the *in vitro* response of the cultured embryos also could be influenced by external as well as internal factors like moisture content etc. (Kitajima, 2007). During the present study attempt was made to test the water stress tolerance of *C. tamala* seeds and embryos. Initially the mature seeds were dehydrated at different level and tested for their germination in the seed bed. It was found that mature seeds dehydrated <60% (fresh weight basis) declined the germination significantly showing the recalcitrant nature of the *C. tamala*

seeds. To confirm the same, the mature seeds were desiccated at different levels and embryos were cultured on optimum initiation medium. The seeds dehydrated up to 80% did not show any significant difference in morphogenetic response but below 80% of fresh weight of the embryos the *in vitro* response declined and desiccation below 50% reduced the response significantly and at 40% moisture level, morphogenetic response dropped to 12% and there was no response at moisture content of 30%. The lowest moisture content withstood by the recalcitrant seeds seems to vary with the species (Barbedo and Cicero, 2000). The finding of the present study clearly shows the recalcitrant nature of the *C. tamala* seeds.

Initiation of Cultures from Cotyledonary Segments, Leaf Explants and Nodal Segments

***Seasonal effect of explants collection and priming*:** *Cinnamomum tamala* is a spice yielding plant and rich in many secondary metabolites including phenolics. For successful culture initiation from both nodal explants as well as foliar explants, it was necessary to control the exudation of phenolic compounds in the medium. The first objective towards the establishment of *in vitro* regeneration protocol for *C. tamala* was to optimize the season of nodal explants collection from the field grown plants. The nodal segments were soaked in different antioxidant solutions immediately after collection and soaked in distilled water after sterilization till cultured on nutrient medium. Soaking of nodal explants in water improved the morphogenetic response over non-soaked segments. The pre-soaked explants released comparatively less phenolics in the medium in comparison to non-soaked segments. Earlier Lakshmanan *et al.* (2006) in sugar cane leaf culture and Deb and Arenmongla (2012) in *Strobilanthes flaccidifolious* observed that soaking of leaf and nodal explants after excision promoted morphogenetic response *in vitro*.

Immediately after harvest, the nodal explants were soaked in different antioxidant solutions. Of the different solutions, citric acid (100 mg L^{-1}) soaked explants supported healthy morphogenetic response. During this priming period, the phenolic compounds and other secondary compounds released in the antioxidant solution. Earlier Deb and Tandon (2004b) treated the apical dome section at 4^{o}C for 24 hr on mMS medium containing 0.4% AC before transferring to initiation medium for raising successful embryogenic culture of *Pinus kesiya*. While, in *Strobilanthes flaccidifolious* nodal explants the sterilized explants precultured on a 'Growtak Sieve' containing PVP (200 mg L^{-1}) suppoted better morphogenetic response as compared to control (Deb and Arenmongla, 2012).

Round the year, the nodal explants were collected at one month interval starting from January till December. In the present study with *C. tamala,* seasonal changes greatly influenced the explants establishment. Generally, the actively growing season is known to be more responsive for bud break (Dhar and Upreti, 1999) which is contrary to the present report, where maximum establishment was achieved just before the onset of winter season. It was observed that amongst the different collection seasons studied, the nodal explants collected during January-May and September-October were least responding and tissues turned necrotic. While, explants collected during June-July responded optimally where as much as ~45% nodal explants responded by sprouting the axillary buds. This is probably due to the fact that during this period due to favorable rainfall and temperature explants responded optimally. Present observation is in contradiction with Deb and Arenmongla (2012) where they reported that the nodal explants collected after the end of the rainy season supported optimum response in *Strobilanthes flaccidifolious*. Mangal *et al*. (2008) in Guava nodal explants culture reported the effect of time

of year on culture initiation and reported that explants collected during February responded optimally under culture condition. While, in *Embelia ribes* minimum culture contamination and less browning of explants was observed when explants were collected during March to May (Dhavala and Rathore, 2010).

Effects of antioxidants: In woody species exudation from of the explants and browning of medium is a common problem and is generally considered due to oxidation of phenolic compounds released from the cut ends of the explants. In the present study, release of phenolic and browning of medium was one of the bottlenecks for successful establishment of culture from all the explants like leaf, cotyledons, and nodal explants. The release of phenolic and browning of medium could be prevented by incorporating antioxidant like PVP (100 mg L^{-1}). Deb and Tandon (2004b) could successfully control the browning of medium by incorporating 200 mg L^{-1} PVP and 100 mg L^{-1} citric acid in combination but, in *Embelia ribes* incorporation of PVP did not reduce the browning of the medium (Dhavala and Rathore, 2010). Gupta (1980) reported that in case of shoot tip culture of apple and teak, agitating the plant materials for an hour or incorporation of PVP and β-mercaptoethanol (0.5-2%) helped in preventing oxidation of phenolic compounds. While, Narayanaswamy (1994) discussed that culture medium fortified with antioxidant like ascorbic acid and citric acid (500-2000 mg L^{-1}) could curtailed the effects of phenolic exudation.

Effects of organic carbon sources: The growth and multiplication of shoot *in vitro* are affected by many factors, one of which is the quality and quantity of exogenously supplied carbon source added to the medium (Anwar *et al.*, 2005). The carbon sources serves as energy and osmotic agents to support the growth of plant tissues. The process of *in vitro* culture establishment is a

highly energy requiring processes that can occur at the expense of available metabolic substrates like organic carbon sources. In the present study, quality and quantity of organic carbon sources in the initiation medium had pronounced effect on *in vitro* morphogenetic response from nodal explants. For culture initiation from nodal explants, three different organic carbon sources at differential concentrations (viz., dextrose, glucose and sucrose at 0-4%) were incorporated in the medium. In absence of organic carbon source, all explants degenerated. Optimum response was recorded on medium containing sucrose (3%) while other carbon sources did not support optimal culture initiation. A similar response was also recorded with foliar explants. Of the different concentrations of sucrose tested, better morphogenetic response was achieved on medium fortified with sucrose (3%). Medium containing higher concentration of sucrose (4%), explants turned brown while, at lower concentrations, fewer shoot buds formed. Earlier, the effect of organic carbon source on *in vitro* morphogenetic response was described in *Stevia rebaudiana* (Preethi *et al.*, 2011), in *Solanum nigrum* (Sridhar and Naidu, 2011) and in *Pogostemon cablin* (Swamy *et al.*, 2010). The requirements of the quality and quantity of exogenous supply of the organic carbon sources vary with the species, the media compositions used, the endogenous level of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005c, Pongener and Deb, 2009, 2011a). In *Cymbidium elegans* and *Coelogyne punctulata* 2-3% sucrose, D-Fructose and D-Glucose were found to suitable for culture initiation (Sharma and Tandon, 1990) while, 3% sucrose was found to be suitable for immature seed germination of *Geodorum densiflorum* (Bhadra and Hossain, 2003). In *Solanum nigrum* nodal explants culture, presence of fructose (4%) in the initiation medium was found to be optimum where as many as 24 shoot buds formed from each node followed by sucrose (4%) and glucose was found to be least preferred (Sridhar and Naidu, 2011).

Effects of PGRs: In all the three explants types, incorporation of at least one of the PGRs was pre-requisite for *in vitro* culture initiation. On PGRs control medium the cultured cotyledons showed only swelling but degenerated subsequently without any morphogenetic response. Of the three PGRs incorporated in different concentrations and combinations, optimum response was registered on BA (6 µM) enriched medium where as many as 6.6 shoots buds developed per cotyledons in ~43% explants followed by TDZ (9 µM, 5.6 shoot buds per cotyledon) (Table 8). Swelling of the axillary buds was observed within a week followed by differentiated into multiple shoot buds/micro shoots formation from nodal segments while, within one wk of culture the cultured foliar explants started swelling followed by callus formation. In the present study, presence of cytokinin was obligatory for inducing morphogenetic response in both the explants. Many stimuli are communicated across the plant body by PGRs which consequently play an important role in diverse aspects of plant growth and development (Mereier *et al.*, 2003, Deb and Arenmongla, 2012). At a cellular level, auxin affects division, expansion and differentiation. Cytokinins are necessary in concerts with auxin in many cases for cell division at G1-S and G2-M transitions in a variety of cultured plant cells as well as in plants. Progression through the cell cycle is central to cell proliferation and fundamental to growth and development of higher plants (Stals and Inze, 2001; Mereier *et al.*, 2003; Abhyankar and Reddy, 2007; Dhavala and Rathore, 2010; Mir *et al.*, 2011).

Of the three PGRs incorporated at differential concentrations for culturing nodal explants, Kn in general found to be inferior over BA either singly or in combination with NAA. Under the given conditions the optimum response was registered on MS medium fortified with BA (9 µM) (Table 13). Earlier Britto *et al*. (2003) and Karuppusamy *et al*. (2007) also reported

the synergistic effect of NAA and BA on nodal explants culture of *Ceropegia bulbosa* and *Hydrocotyle conferta* respectively. Dhavala and Rathore (2010) reported that cytokinin alone could not promote axillary bud breaking in *Embelia ribes* unless one of the auxin especially IAA is incorporated in the medium in conjunction with cytokinin. While in Guava nodal segment culture, incorporation of GA_3 along with BA was prerequisite for axillary bud breaking (Mangal *et al*., 2008). But in *Adhatoda vasica* Nees. axillary bud proliferation and multiple shoot initiation were optimum on MS medium containing BA alone (Abhyankar and Reddy, 2007). In case of sugarcane leaf culture, NAA and BA in combination promoted shoot bud formation but NAA alone induced somatic embryogenesis (Lakshmanan *et al*., 2006).

For leaf explants singly use of BA across the concentrations failed to invoke any response and Kn enriched medium supported partial swelling of the explants. When both BA and Kn were conjunct with NAA, resulted into callus formation and optimum callus (~28%) formation was registered on MS medium fortified with sucrose (3%), BA and NAA (6 and 3 µM respectively) in combination. Lakshmanan *et al.* (1997) noted that the cytokinin (BA) treatment is required during the induction period of six days for fully competent cells to enter into a caulogenically determined state in leaf explants of *Gracinia mangostana*. In *Stevia rebaudiana* leaf culture, a combination of BA and IAA; BA and NAA promoted multiple shoot buds formation (Sridhar and Naidu, 2011).

Plant Regeneration and Culture Proliferation

The shoot buds/micro shoots developed on initiation medium from the cultured zygotic embryos (direct morphogenesis and callus mediated), leaf callus, cotyledons and nodal segments

were maintained for another two passages. The micro shoots are then maintained on different strengths of MS medium containing different levels of sucrose (0-4%) as organic carbon sources and different PGRs.

Effect of strength of basal medium: Amongst the different strengths of MS medium tested full strength MS medium supported highest numbers of plantlets formation and better culture proliferation followed by 3/4th strength and 1/2 strength. At lower strength of MS medium (1/4th and 1/2 strengths) most of the plantlets were either stunted in growth with fewer new shoot buds formation or cultures degenerated. At 3/4th strength of MS medium the plantlet height was comparatively better than 1/4th and 1/2 strength media. In this condition, though regenerated plants were healthy with well developed roots but supported fewer shoot buds formation. This perhaps could be due to difference of chemical constituents with MS medium or deficient as to the requirement of the developing shoot buds. Chen *et al.* (2004), George and Sherrington (1984), Temjensangba and Deb (2005a), Pongener and Deb (2009, 2011a) argued that the change in culture conditions and media could alter the pattern of organogenesis and such behavior can be judiciously exploited to achieve desirable response in many taxa by altering the nutrient regime.

Effect of quantity of sucrose: Sucrose at various concentrations (0-4%) was incorporated in the regeneration medium. Incorporation of sucrose was obligatory for regeneration and mass multiplication of plantlets. There was no regeneration in the absence of sucrose. The requirement of the quality and quantity of exogenous supply of the organic carbon sources varies with the species, the media compositions used, the endogenous level of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005a, c, Pongener and

Deb, 2011a). Sharma and Tandon (1990) reported that 2-3% of sucrose; D-Fructose and D-Glucose were the suitable organic carbon sources for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata*. In the present investigation, amongst the different concentrations of sucrose studies, optimum regeneration and shoot bud formation in *C. tamala* was obtained on basal media containing sucrose (3%).

Effects of PGRs: For regeneration of plantlets, incorporation of one of the PGRs was obligatory. In the absence of PGRs all the cultures degenerated. Amongst the two PGRs tested, BA in the entire range did not support optimum culture proliferation. When BA tested singly, at concentration of 3 µM supported 3 shoot bud formation after 8 wk of culture where plant height was stunted (~3.2 cm) with only two roots. But incorporation of Kn singly proved to be superior for shoot proliferation, root formation and plant height. About 5 shoot buds with an average of 8 roots were formed on medium containing Kn (3 µM) where average plant height was ~4.5 cm. While BA and Kn in combination exhibited a more or less similar response in the entire range studied and did not support optimum plant regeneration and culture proliferation.

The effectiveness of cytokinin on plant regeneration and culture proliferation is reported by many earlier workers (Selvaraj *et al*., 2006, Baskaran *et al*., 2009). In *Acacia confuse*, BA, NAA and Kn in combination (0, 0.05 and 0.05 mg L^{-1} respectively) produced maximum shoot buds where as many as 25 shoot buds developed in culture (Arumugam *et al*., 2009).

Rooting, Hardening and Transplantation to Potting Mix

Though there were some roots formation in some regenerated shoots on regeneration medium, but roots were not fully developed and shorter in length. Regenerated shoots (~4-5 cm

in length, sources directly from regeneration medium) induced roots when transferred on rooting medium. The shoots were treated differentially for inducing roots. Under optimum condition, only 10 roots were formed after ~5 wk of culture on MS medium fortified with NAA (3µm). The role of auxin in rooting of the *in vitro* raised micro shoots have been shown in several species, including apple (Moncousin *et. al.*, 1992), *Quercus suber* (Manzanera and Pardos, 1990), *Strobilanthes flaccidifolius* (Deb and Arenmongla, 2012). Auxins have been shown to act as a local morphogenetic trigger on the formation of lateral roots in *Arabidopsis*, leading to the specification of founder cells of the new organ from previous differentiated cells (Dubrovsky *et al.*, 2008). The promotory effect of NAA on rooting is also described in rice (Biswas and Mandal, 2007), in *Populus euphratica* (Ferreira *et al.*, 2009). It was reported that NAA was better option followed by IAA and IBA. While, in some other reports, IBA was found to be superior over other PGRs for rooting (Abhyankar and Reddy, 2007; Baskaran *et al*., 2009, Arumugam *et al*., 2009).

The rooted plants were hardened on medium with ½MS salt solution containing sucrose (2%) and maintained for 6-7 wk under normal laboratory condition. The hardened plants were transferred to plastic pots as mentioned in the materials and methods. The plants were successfully transferred to a shade-house and then to field. About 200 plants were tested for survival and about 70% survival was registered after two months of transfer.

3.4. Conclusion

During the present investigation, protocols were established for culture initiation from immature zygotic embryos, leaf explants, cotyledons and nodal explants from *in vivo* source.

These techniques open new routes for *in vitro* mass multiplication of this economically important spice yielding species. The protocols established for culture initiation from cotyledons, foliar explants and nodular explants indicates the possibility of using alternative explants. The protocols may be used for production of clonal planting material in commercial scale and could be used by the spice industry.

Chapter - 4

Summary

Cinnamomum tamala (Buch.-Hum) T. Nees. & Eberm (Lauraceae) is an evergreen medium-sized tree found along with North-Western Himalayas, North Eastern region of India. *C. tamala* is a multipurpose plant used for medicines, spices, natural dyes etc. The leaves of *C. tamala* are widely used as spice and also source of oil (tejpat oil) which is medicinally used as carminative, anti-flatulent, diuretic and in cardiac disorder. '*Tejpatra*' is also used in different '*Ayurvedic'* medicine preparation. Owing to its high medicinal value and being an important ingredient of the spices the demand of *C. tamala* is increasing day by day and the species being exploited from its natural pockets illegally. According to the recent report, *C. tamala* has been found vulnerable in throughout the Himalayan region and North Eastern states and endangered in Jammu and Kashmir. Therefore, there is a need to raise high quality individuals in large scale

to fulfill the increasing demand in one hand and help the conservation of the species on the other hand. However, in the absence of standard agro-techniques and owing to lack of information on seed germination behaviors, conservation efforts have not succeeded so far. In view of these, the present study was under taken on seed biology and development of culture systems for production of clonal planting materials.

During the present investigation, the mature fruits were collected and seeds were extracted. The seeds were desiccated at various levels (100 to 30% fresh weights) following rapid and slow drying methods to test their desiccation tolerance. Besides desiccation tolerance, effect of microclimatic conditions like light intensity on seed germination, seed storage temperature and duration on seed germination were also tested. Within 10 days of sowing, radical started emerging from the germinated seeds. Of the different light conditions tested, 50% shading in the seed bed supported highest germination and healthy seedling growth in comparison to full light and 75% shading and under optimum condition ~72% seeds germinated.

In the present study, the seeds could be desiccated up to 80% (fresh weight) without significant change in the germination rate. The seeds desiccated below 70%, germination rate decreased significantly and desiccation below 40% did not support any germination. The seed stored for variable period at 4^{o}C and 25^{o}C before sowing in the seed beds. The seeds could not be stored beyond 4 days at 4^{o}C without effecting their germination. The findings of this study clearly indicate that the *Cinnamomum tamala* seeds are desiccation sensitive and recalcitrant in nature.

For developing *in vitro* culture systems, different explants like embryos of various developmental stages, foliar explants, nodal segments and cotyledonary segments were used. Successful morphogenetic response (~60%) was achieved from embryos of 12 week after flowering (WAF) on MS medium fortified with different adjuncts. The embryo age <12 WAF failed to give significant response. The embryos were also desiccated at various levels before culture to confirm the desiccation sensitivity which was observed from the seed germination experiments in the seed beds. A similar response was also recorded with the *in vitro* culture where seeds desiccated below 70% (fresh weight) reduced the morphogenetic response and confirms the desiccation intolerance character of *C. tamala* seeds. Of the different strengths of MS medium, organic carbon sources and plant growth regulators incorporated, optimum morphogenetic response was achieved on full strength MS medium fortified with sucrose (3%, w/v) and BA (12 µM). While, the cultured cotyledons started swelling and invoked meristematic loci within two week of culture. Of the different culture conditions provided, shoot buds developed in ~43% explants on MS medium fortified with sucrose (3%), BA (6 µM).

The newly flushed shoots were collected in the month of July and nodal segments were cultured on different strengths of MS medium adjuncts with different supplements. Shoot buds invoked from the cultured nodal explants on MS medium conjunct with sucrose (3%), PVP (100 mg L^{-1}) and BA (9 µM) where ~45% explants responded positively. Under optimum condition, as many as 5.4 shoot buds developed per node after 4-5 wk of culture. The shoot buds developed from the different explants were maintained for another 2 passages at 4-5 wk interval for further growth and differentiation.

The cultured foliar explants developed callus on MS medium enriched with sucrose (3%) and BA (6 µM) and NAA (3 µM) where ~28% explants callused within two week of culture. Initiation of callusing started at the basal part which subsequently spread the entire leaf. The intact leaf performed better over the leaf segments. The calli developed from the zygotic embryos, nodal segments and foliar explants were maintained for two more passages on the respective optimum initiation media for further proliferation. Thereafter the calli were transferred on MS medium supplemented with sucrose (3%), BA (6 µM) and maintained for two passages where shoot buds developed.

The resultant shoot buds were maintained on regeneration medium for plantlet formation and culture proliferation. The micro shoots, shoot buds developed on MS medium fortified with sucrose (3%), kinetin (3 µM) where ~5 shoot buds developed per sub-culture. The micro shoots (size: ~4.5 cm) were rooted on MS medium enriched with NAA (3 µM) followed by hardened for 6-7 wk. The hardened plants were transferred to potting mix and maintained in the poly house (75% shade) for two months followed by transferring to the natural habitat. About 70% of the transplants survived after two months of transfer.

During the present investigation, protocols were established for culture initiation from immature zygotic embryos, leaf explants, cotyledons and nodal explants from *in vivo* source. These techniques open new routes for *in vitro* mass multiplication of this economically important spice yielding species. The protocols established for culture initiation from cotyledons, foliar explants and nodular explants indicates the possibility of using alternative explants. The protocols may be used for production of clonal planting material in commercial scale and could be used by the spice industry.

References

Abdul Karim, A. G. and Hairani, H. 1990. Leaf culture of some Malaysian orchids. In: *Proc. International Conference and Exhibition on Orchids and Ornamental Plants.* Kuala Lumpur, Malaysia. Pp. 12.

Abhyankar, G. and Reddy, V. D. 2007. Rapid micropropagation via axillary bud proliferation of *Adhatoda vasica* Nees from nodal segments. *Indian J Exp Biol*, 45: 268-271.

Andrews, H. Y.; Cadwalleder, K. R.; Grodner, R. M. and Chung, H. Y. 1995. Chemical and microbial quantity of irradiated ground ginger. *J Food Sci*, 60(4): 829-832.

Anonymous. 1950. Wealth of India. PID, CSIR, New Delhi, India.

Anonymous. 2006. Agricultural marketing. Statistical abstract. *National School of Agricultural Marketing*. Kota road, Bambala, Jaipur, India.

Anwar, Md. H.; Hossain, Md. T.; Raihanali, Md. and Rahman, S. M. M. 2005. Effect of different carbon sources on *in vitro* regeneration of Indian Penny wort (*Centella Asiatic* L.). *Pak J Biol Sci*, 8(7): 963-965.

Arditti, J. and Ernst, R. 1993. Micropropagation of Orchids. *John Wiley and Sons Inc.*, New York.

Arumugam, S.; Chu, F. H.; Wang, S. Y. and Chang, S. T. 2009. *In vitro* plant regeneration from immature leaflets derived callus of *Acacia confusa* Merr via organogenesis. *J Pl Biochem Biotech*, 18(2): 197-201.

Barbedo, C. J. and Filho, J. M. 1998. Desiccation tolerance on seeds. *Acta Botanica Brasilica*, 12: 145-164.

Barbedo, C. J. and Cicero, S. M. 2000. Effects of initial quality, low temperature and ABA on the storage of seeds of *Inga uruguensis*, a tropical species with recalcitrant seeds. *Seed Sci Technol*, 28: 793-808.

Baskaran, P.; Velayutham, P. and Jayabalan, N. 2009. *In vitro* regeneration of *Melothria maderaspatana* via indirect organogenesis. *In Vitro Cell Dev Biol Pl*, 45: 407-413. DOI 10.1007/s11627-008-9172-8.

Baskin, C. C. and Baskin, J. M. 1988. Germination ecophysiology of herbaceous plant species in a temperature region. *Ame J Bot*, 75: 286-305.

Berjak, P. 2005. Protector of seeds: seminal reflections from Southern Africa. *Science*, 307: 47-49.

Berjak, P. and Pammenter, N. W. 1994. Recalcitrant is not an all-or-nothing situation. *Seed Sci Res*, 4: 263-264.

Berjak, P.; Farrant, J. M.; Mycock, D. J. and Pammenter, N. W. 1990. Recalcitrant (homeohydrous seeds): the enigma of their desiccation-sensitivity. *Seed Sci Technol*, 18: 297-310.

Bhadra, S. K. and Hossain, M. M. 2003. *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Schltr., an endangered orchid species. *Pl Tiss Cult*, 13: 165-171.

Bhat, S. R. and Chandel, K. P. S. 1991. A novel technique to overcome browning in tissue culture. *Pl Cell Rep*, 10: 35-361.

Biswas, A. and Mandal, A. B. 2007. Plant regeneration of different genotypes of *Indica* rice. *Indian J Biotech*, 6: 532-540.

Britto, S. J.; Natarajan, E. and Arockiasamy, D. I. 2003. *In vitro* flowering and shoot multiplication from nodal explants of *Ceropegia bulbosa* Roxb. var. bulbosa. *Taiwania*, 48: 106-111.

Cahkraborty, U. and Das, H. 2010. Antidiabetic and antioxidant activities of *Cinnamomum tamala* leaf extracts in Stz –treated diabetic rats. *Global J Biotech Biochem*, 5(1): 12-18.

Chaturvedi, H. C. and Sharma, A. K. 1986. Mericloning of orchids through culture of tips of leaves and roots. (ed. Vij, S. P.) *Biology, Conservation and Culture of Orchids.* Affiliated East-West Press, New Delhi, India. Pp. 469-472.

Chen, T. Y.; Chen, J. T. and Chang, W. C. 2004. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. *Pl Cell Tiss Org Cult*, 76: 11-15.

Chang, S. T. and Cheng, S. S. 2002. Antitermite activity of leaf essential oils and components from *Cinnamomum osmophleum*. *J Agri Food Chem*, 50(6): 1389-1392.

Cahudhry, N. M. A. and Tariq, P. 2006. Anti-microbial activities of *Cinnamomum cassia* against diverse microbial flora with its nutitional and medicinal impacts. *Pak J Bot*, 38(1): 169-174.

Chin, H. F.; Roberts, E. H. 1980. Recalcitrant crop seeds. Tropical Press, SDN BDH, Kuala Lampur, Malaysia.

Chin, H. F.; Hor, Y. I. and Mohd Lassim, M. B. 1994. Identification of recalcitrant seeds. *Seed Sci Technol*, 12: 429-436.

Corredoira, E.; Vieitez, A. M. and Ballester, A. 2002. Somatic embryogenesis in Elm. *Annl Bot*, 89: 637-644.

Cuenca, B.; San-Jose, M. C.; Martinez, M. T.; Ballester, A. and Vieitez, A. M. 1999. Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. *Pl Cell Rep*, 18: 538-543.

Cunha, R.; Eira, M. T. S. and Rita, I. 1995. Germination and desiccation studies on wild nutmeg seed (*Virola surinamensis*). *Seed Sci Technol*, 23: 43-49.

Das, P.; Samantaray, S.; Roberts, A. V. and Rout, G. R. 1997. *In vitro* somatic embryogenesis of *Dalbergia sissoo* Roxb. – a multipurpose timber-yielding tree. *Pl Cell Rep,* 16: 578-582.

Davies, S. J. 2001. Tree mortality and growth in 11 sympatric *Macaranga* species in Borneo. *Ecology*, 82: 920-932.

Daws, M. I.; Lydall, E.; Chmierlaz, P. Leprince, O.; Matthews, S.; Thanos, C. A. and Pritchard, H. W. 2004. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. *New Phytol*, 162: 157-166.

Deb, C. R. 2001. Somatic embryogenesis and plantlet regeneration of *Melia azedarach* L (Ghora neem) from cotyledonary segments. *J Pl Biochem Biotech*, 10: 63-65.

Deb, C. R. and Arenmongla, T. 2012. An efficient *in vitro* regeneration protocol for a natural dye yielding plant, *Strobilanthes flaccidifolious* Nees., from nodal explants. *Indian J Exp Biol*, 50(11) (In Press).

Deb, C. R. and Sungkumlong. 2008. *In vitro* regeneration and mass multiplication of *Taenia latifolia* (Lindl.) using immature seeds: A threatened teristrial orchid. *J Pl Biol*, 35: 1-6.

Deb, C. R. and Sungkumlong. 2009. Rapid multiplication and induction of early *in vitro* flowering in *Dendrobium primulinum* Lindl. *J Pl Biochem Biotech*, 18: 241-244.

Deb, C. R. and Sungkumlong. 2010. Regenerative competence of foliar explants of *Coelogyne suaveolens* and *Taenia latifolia*: two threatened orchids of North-East India. *Appl Biol Res*, 12: 1-9.

Deb, C. R. and Tandon, P. 2002a. Induction of somatic embryogenesis in Khasi pine (*Pinus kesiya*) from secondary needles. *J Pl Biol*, 29: 113-118.

Deb, C. R. and Tandon, P. 2002b. Somatic embryogenesis and plantlet regeneration from mature embryos of *Pinus kesiya* (Royle ex. Gord). *J Pl Biol,* 29: 301-306.

Deb, C. R. and Tandon, P. 2004a. Establishment of an embryogenic suspension culture of *Pinus kesiya* (Khasi pine) from various explants. *Indian J Biotech,* 3: 445-448.

Deb, C. R. and Tandon, P. 2004b. Factors influencing initiation of embryogenic cultures in *Pinus kesiya* Royle ex Gord. *Indian J Biotech,* 3: 589-593.

Deb, C. R. and Temjensangba. 2005. *In vitro* regenerative competence of *Cleisostoma racemiferum* (Orchidaceae) aerial roots. *J Pl Biochem Biotech*, 14: 201-205.

Deb, C. R. and Temjensangba. 2006a. On the regeneration potential of *Arachnis labrosa* (Lindl. Ex. Paxt.) Reichb. Root segments: A study *in vitro*. *Phytomorphology,* 56: 79-83.

Deb, C. R. and Temjensangba. 2006b. *In vitro* propagation of threatened terrestrial orchid, *Malaxis khasiana* Soland ex. Swartz through immature seed culture. *Indian J Exp Biol,* 44: 762-766.

Deb, C. R. and Temjensangba. 2007a. Direct regeneration of shoot buds in *Arachnis labrosa* foliar explants. *J Orchid Soc India,* 21: 7-9.

Deb, C. R. and Temjensangba. 2007b. Rapid mass multiplication of *Cleisostoma racemiferum* (Lindl.) Garay: An endangered orchid. *J Pl Biol,* 34: 99-105.

Deb, C. R.; Deb, M. S.; Jamir, N. S. and Imchen, T. 2009. Orchids in indigenous system of medicine in Nagaland, India. *Pleione*, 3(2): 209-211.

Devi, J.; Nath, M.; Devi, M. and Deka, P. C. 1990. Effect of different media on germination and growth of some North East Indian species of *Dendrobium*. *J Orchid Soc India*, 4: 45-49.

Dhar, U. and Upreti, J. 1999. *In vitro* regeneration of a mature leguminous liana (*Bauhinia vahlii* Wight & Arnott). *Pl Cell Rep*, 18: 664-669.

Dhar, U.; Manjkhola, S; Joshi, M.; Bhat, A.; Bisht, A. K. and Joshi, M. 2002. Current status and future strategy for development of medicinal plant sector in Uttaranchal, India. *Curr Sci*, 83: 956-963.

Dhavala, A. and Rathor, T. S. 2010. Micropropagation of *Embelia ribes* Burm f. through proliferation of adult axillary shoots. *In Vitro Cell Dev Biol Pl*, 46: 180-191. DOI: 10.1007/s11627-010-9285-8.

Dighe, V. V.; Gursale, A. A.; Sane, R. T.; Menon, S. and Patel, P. H. 2005. Quantative determination of Eugenol from *Cinnamomum tamala* Nees and Eberm. leaf powder and polyherbal formulation using reverse phase liquid chromatography. *Chromatographia*, 61: 443-446.

Dikshit, V. K. 1999. Export of medicinal plants from India: need for resource management. In: (eds. *Biodiversity – North East India perspectives : people's participation inbiodiversity conservation*. NEBRC, North Eastern Hill University, Shillong, India. Pp 85-88.

Dubrovsky, J. G.; Sauer, M.; Napsucialy-Mendivil, S.; Ivanchenko, M. G.; Friml, J.; Shishkova, S.; Celenza, J. and Benkova, E. 2008. Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. In: *Proc Natl Acad Sci, USA*, 105(25): 8790-8794. DOI: 10.1073/pnas.0712307105.

FAO. 1995. Food and Agriculture Organization of the United Nations. Chapter 2 –*Cinnamomum* oils (including *Cinnamomum* and *Cassia*). 'Flavours and fragrances of plant origin' Non-Wood Forest Products 1. FAO – M-37, ISBN 92-5-103648-9,(c) FAO.

Farrant, J. M.; Pammenter, N. W. and Berjak, P. 1993. Seed development in relation to desiccation tolerance: a comparision between desiccation sensitive (recalcitrant) seds of *Avicennia marina* and desiccation tolerant types. *Seed Sci Res*, 3: 1-13.

Ferreira, S.; Batista, D.; Serrazina, S. and Pais, M. S. 2009. Morphogenesis induction and organogenic nodule differentiation in *Populus euphratica* Oliv. leaf explants. *Pl Cell Tiss Org Cult*, 96: 35-43. DOI: 10.1007/s11240-008-9457-y.

Finch-Savage, W. E.; Balke, P. S. and Clay, C. A. 1996. Desiccation stress in recalcitrant *Quercus robur* L. seeds results in lipid peroxidation and increased systhesis of jasmonate and ascorbic acid. *J Exp Bot*, 47; 661-667.

Fischer, I. U.; Dengler, I. J. 1990. Sensitive high performance liquid chromatography assay for the determination of eugenol in body fluids. *J Chromatography*, 525: 369-377.

Fu, J. R.; Zhang, B. Z.; Wang, X. P.; Qiao, Y. Z. and Huang, X. L. 1990. Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three fruit species and their excised embryonic axes. *Seed Sci Tech*, 18: 743-754.

Gaur, R. D. 1999. Flora of district Garhwal: North West Himalaya (with ethnobotanical notes). Thran Media, Srinagar, Garhwal, India.

Gaur, R. D. 2008. Traditional dye yielding plants of Uttarakhand, India. *Natural Products Radiance,* 27(2): 154-165.

George, E. F. and Sherrington, P. D. 1984. Plant propagation by tissue culture: Handbook and Directory of Commercial Laboratories. *Exegetics*, Hants.

Godo, T.; Komori, M.; Nakaoki, E.; Yukawa, T. and Miyoshi, K. 2010. Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid by asymbiotic culture *in vitro*. *In Vitro Cell Dev Biol Pl*, 46: 323-328. DOI: 10.1007/s11627-009-9271-1.

Gupta, P. K. 1980. Plant regeneration of apple and teak through shoot tip culture. *Pl Sci Lett*, 17: 259-268.

Hadacek, F. 2002. Secondary metabolites as plant traits: current assessment and future prespective. *Crit Rev Pl Sci*, 21: 273-322.

Holm, L.; Doli, J.; Holm, E.; Pancho, J. and Herberger, J. 1996. World weeds. Natural histories and distribution. John Wiley and Sons Inc. NY.

Johnson, T. R.; Stewart, S. L.; Dutra, D.; Kane, M. E. and Richardson, L. 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)-preliminary evidence for the symbiotic culture advantage. *Pl Cell Tiss Org Cult,* 90: 313-323. DOI: 10.1007/s11240-007-9270-z.

Kapoor, L. D. 2000. CRC Handbook of Ayurvedic Medicinal Plants. CRC Press, Inc. Boca Raton, Florida, Pp. 117.

Kapoor, I. P. S.; Singh, B.and Singh, G. 2008. Essential oil and oleoresins of *Cinnamomum tamala* (tejpat) as natural food preservatives for pineapple fruit juice. *J Food Processing Preserv*, 32(5): 719-728.

Karuppusamy, S.; Aruna, V.; Kiranmai, C. and Pullaiah, T. 2007. *In vitro* propagation of an endemic umbellifer, *Hydrocotyle conferta*. *Indian J Biotech,* 6: 541-544.

Katiyar, R. S.; Sharma, G. D. and Mishra, R. R. 1987. Asymbiotic seed germination and seedling development in *Coelogyne punctulata* and *Aerides multiflorum*. *Indian Forester,* 113: 574-577.

Kermode, A. R. 1990. Regulatory mechanisms involved in the transition from seed development to germination. *Critical Rev Pl Sci*, 9: 155-195.

Kirtikar, K. R. and Basu, B. D. 1981. Indian medicinal plants (2nd edition), Lalit Mohan Basu Publisher, Ahmadabad, India. Pp. 2146-2147.

Kitajima, K. 2007. Seed and seedling ecology. In: (eds. Pugnaire, F. I. and Valladares, F.) Functional Plant Ecology, 2nd Edn. CRC Press, Tylor & Francies Group. pp. 549-580.

Knudson, L. 1946. A new nutrient solution for germination of orchid seeds. *Amer Orchid Soc Bull*, 15: 214-217.

Kwit, C. and Platt, W. J. 2003. Disturbance history influences regeneration of non-pioneer understory trees. *Ecology*, 84: 2575-2581.

Lakshmanan, P.; Ng, S. K.; Loh, C. S. and Goh, C. J. 1997. Auxin, cytokinin and ethylene differentially regulate specific developmental states associated with shoot bud morphogenesis in leaf tissues of mangostane (*Gracinia mangostana* L.) cultured *in vitro*. *Pl Cell Physiol*., 38: 59-64.

Lakshmanan, P.; Geijskes, R. J.; Wang, L.; Elliott, A.; Grof, C. P. L.; Berding, N. and Smith, G. R. 2006. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrid) leaf culture. *Pl Cell Rep*, 25:1007-1015. DOI 10.1007/s00299-006-0154-1.

Laloo, R. C.; Kharlukhi, L.; Jeeva, S. and Mishra, B. P. 2006. Status of medicinal plants in the disturbed and the undisturbed sacred forest of Meghalaya, North East India: population structure and regeneration efficacy of some important species. *Curr Sci*, 90: 225-232.

Li, Z. and Xu, L. 2009. *In vitro* propagation of white-flowered mutant of *Rhynchostylis gigantean* (Lindl.) Ridl. Through immature seed-derived protocorm-like bodies. *J Hortic For*, 1: 93-97.

Mandal, A. K. A.; Chatterji, A. K. and Dutta, G. S. 1995. Direct somatic embryogenesis and plantlet regeneration from cotyledonary leaves of safflower. *Pl Cell Tiss Org Cult,* 43: 287-289.

Mangal, M.; Sharma, D.; Sharma, M.; Kher, R. and Singh, A. K. 2008. *In vitro* plantlet regeneration in Guava from nodal segments. *Phytomorphology* 58: 103-108.

Manzanera, J. A. and Pardos, J. A. 1990. Micropropagation of juvenile and adult *Quercus suber* L. *Pl Cell Tiss Org Cult*, 21: 1-8. DOI: 10.1007/BF00034484.

Mereier, H.; Souza, B. M.; Kraus, J. E.; Hamsaki, R. M. and Sotta, B. 2003. Endogenous auxin and cytokinin contents associated with shoot formation in leaves of pineapple cultured *in vitro*. *Braz J Pl Physiol*, 15: 107 -112.

Mir, F. A.; Yadav, A. S. and Wankhede, S. 2011. Efficiency of media's for propagation of medicinal tree *Gardenia gummifera* Linn. F- An endangered medicinal plant. *J Phytology*, 2(8): 47-51.

Misra, M. K. 1999. Need for conservation of indigenous medicinal knowledge and the herbs. *J Hum Ecol*, 10: 403-406.

Mitra, G. C.; Prasad, R. N. and Roy Chowdhury, A. 1976. Inorganic salt and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J Exp Biol*, 14: 350-351.

Moncousin, C.; Ribaux, M.; O'Rourke, J. and Gavillet, S. 1992. Effects of type of carbohydrate during proliferation and rooting of micro cuttings of *Malus* Jork-9. *Agronomie,* 12:775-781.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Planta,* 15: 473-497.

Murthy, B. N. S. and Saxena, P. K. 1998. Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). *Pl Cell Rep*, 17: 469-473.

Myers, N.; Mittermeler, R. A. and Mittermeler, C. G. 2000. da Fonseca, G.A.B. & Kents, J. (2000). *Nature,* 403: 853-858.

Nagaraju, V.; Das, S. P.; Bhutia, P. C. and Upadhyaya, R. C. 2004. *In vitro* multiplication of *Dendrobium chrysotoxum* and two Dendrobium crosses (*D. nobile* x *D. nobile* var. *alba* and *D. nobile* x *D. heterocarpum*) through embryo culture. *J Orchid Soc India,* 18: 47-51.

Nakagawa, M. and Kurahashi, A. 2005. Factors affecting soil-based natural regeneration of *Abies sachalinensis* following timber harvesting in a sub-boreal forest in Japan. *New Forests*, 29: 199-205.

Narayanaswamy, S. 1994. Plant cell and tissue culture. P. 16. Tata Mc Graw-Hill Publishing Company Ltd. New Delhi, India.

Nath, M.; Devi, J.; Borthakur, B.; Sharma, J. and Deka, P. C. 1991. Embryo culture of *Rhynchostylis retusa* and *Vanda coerulea*. *J Orchid Soc India,* 5: 97-101.

Neves, C. S. V. J. 1994. Recalcitrant seeds: A review. *Pesquisa Agropecudria Brasileira*, 29: 1459-1467.

Nitsch, J. P. and Nitsch, C. 1969. Haploid plants from pollen grains. *Sci*, 163: 85-87.

Ochoa, M. E. S.; Diaz, I. A.; Gomez, A. C. and Garciglia, R. S. 2010. Callus growth and plant regeneration in *Laelia speciosa* (Orchidaceae). *Lankesteriana*, 10(1): 13-18.

Oliveira, L. M. Q. and Valio, I. F. M. 1992. Effects of moisture content on germination of seeds of *Hancornia speciosa* Gom. (Apocynaceae). *Annals Bot*, 69: 1-5.

Onen, H. 1999. Studies on biology and control of mugwort (*Artemisia vulgaris* L.). Ph. D. thesis. Gaziosmanpasa University, Tokat-Turkey.

Pacala, S. W.; Canham, C. D.; Silander, J. A. and Kobe, R. K. 1994. Sapling growth as a function of resources in a north temperate forest. *Canadian J For Res*, 24: 2172-2183.

Pages, J. P.; Pache, D.; Joud, N.; Magnan, N. and Michalet, R. 2003. Direct and indirect effects of shade on four tree seedlings in the French Alps. *Ecology*, 84: 2741-2750.

Pammenter, N. W. and Berjak, P. 1999. A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Sci Res*. 9: 13-37.

Pammenter, N. W. and Berjak, P. 2000. Evaluation and ecological aspects of recalcitrant seed biology. *Seed Sci Res*, 10: 1301-1306.

Pammenter, N. W.; Vertucci, C. W. and Berjak, P. 1991. Homeohydrous (Recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. *Pl Physiol*, 96: 1093-1098.

Pammenter, N. W.; Berjak, P.; Farrant, J. M.; Smith, M. T.; Ross, G. 1994. Why do stored hydrated recalcitrant seeds die? *Seed Sci Res*, 4: 187-191.

Pammenter, N. W.; Graggains, V.; Kioko, J. I.; Wesley-Smith, J.; Berjak, P. and Finch-Savage, W. E. 1998. Effects of different drying rates on viability retention of recalcitrant seeds of *Ekebergia capensis*. *Seed Sci Res*, 8: 463-471.

Pongener, A. and Deb, C. R. 2009. Asymbiotic culture of immature embryos, mass multiplication of *Cymbidium iridioides* D. Don and the role of different factors. *Intl J Pharma Bio Sci*, 1(1):1-14.

Pongener, A. and Deb, C. R. 2011a. *In vitro* regeneration of plantlets of *Cymbidium irridioides* D. Don using nodal segments as explants. *Intl J Appl Biotech Biochem*, 1(4): 389-400.

Pongener, A. and Deb, C. R. 2011b. *In vitro* mass multiplication of *Cymbidium aloifolium* using immature embryos as explant. *J Plant Biochem Biotech*, 20:90-95. doi: 10.1007/s13562-010-0031-4.

Preethi, D.; Sridhar, T. M. and Naidu, C. V. 2011. Direct Shoot Organogenesis from Leaf Explants of *Stevia rebaudiana*. *J Phytology*, 3(5): 69-73.

Pritchard, H. W.; Daws, M. I.; Fletcher, B. J.; Gamene, C. S.; Msanga, H. P. and Omondi, W. 2004. Ecological correlates of seed desiccation tolerance in tropical African dryland trees. *Ame J Bot,* 91: 863-870.

Raghuvanshi, A. N.; Mishra, R. R. and Sharma, G. D. 1986. Effect of *p*H on asymbiotic seed germination and seedling development of orchids. In: (ed. Vij, S. P.) *Biology, Conservation and Culture of Orchids.* Affiliated East-West Press, New Delhi, India. Pp. 453-462.

Raghuvanshi, A. N.; Mishra, R. R. and Sharma, G. D. 1991. Symbiotic seed germination and seedling development of orchids at different temperatures. *J Orchid Soc India,* 5: 61-69.

Rao, A. N.; Ahmed, N. and Hegde, S. N. 1998. Culture notes from Tipi-4: *In vitro* culture of some rare and endangered orchids of Arunachal Pradesh. In: *International Festival of Orchids*. Orchid Society of Arunachal, Arunachal Pradesh, India. Pp. 16-27.

Rao, N. K.; Hanson, J.; Dulloo, M. E.; Ghosh, K.; Nowell, D. and Larinde, M. 2006. Mannual of seed handling in genebanks. Biodiversity International, Rome, Italy.

Rathore, M. S. and Shekhawat, N. S. 2008. Incredible spices of India: from traditions to cuisine. *Ame Eurasian J Bot*, 1(3): 85-89.

Rawat, N.; Sharma, G.; Prasad, P. and Nautiyal, A. R. 2009. Study on accountable factors for physiological and biochemical variations in normal and variant *Cinnamomum tamala* (Nees and Eberm) seedlings. *Nature and Sci*, 7(11): 58-64.

Saino, F.; Ghizzoni, C.; Gionfriddo, F.; Colombo, E.; Servillo, L and Castaldo, D. 2003. Determination of estragole, safrole and eugenol methyl ether in food products. *Food Chem*, 81: 469-475.

Samant, S. S.; Dhar, U. and Palni, L. M. S. 2001. Himalayan medicinal plants: potential and prospects. Gyanodaya Prakashan, Nainital, India.

Sambyal, M.; Dogra, A.; Koul, S. and Ahuja, A. 2006. Rapid *in vitro* propagation of *Potentilla fulgens* Wall – A Himalayan Alpine herb of medicinal value. *J Pl Biochem Biotech,* 15: 143-145.

Sarin, Y. K. 2008. *Principal crude herbal drug of India*. Bishen Singh Mahendra Pal Singh, Dehra Dun, India.

Sauleda, R. P. 1976. Harvesting time for orchid seed capsule for the green pod culture process. *Amer Orchid Soc Bull*, 45: 305-308.

Seeni, S. and Latha, P. G. 1992. Foliar regeneration of endangered red Vanda *Renanthera imschootiana* Rolfe (Orchidaceae). *Pl Cell Tiss Org Cult,* 29: 167-172.

Selvaraj, N.; Vasudevan, A.; Manickavasagam, M. and Ganapathi, A. 2006. *In vitro* organogenesis and plant formation in cucumber. *Bio Pl,* 50: 123-126. DOI 10.1007/s10535-005-0085-7.

Sharma, G. and Nautiyal, A. R. 2009. Influence of explants types and plant growth regulators on *in vitro* multiple shoots regeneration of a Laurel from Himalaya. *Nature Sci*, 7(9): 1-7.

Sharma, G. and Nautiyal, A. R. 2011. *Cinnamomum tamala*: A valuable tree from Himalayas. *Intl J Med Arom Pl*, 1(1): 1-4.

Sharma, S. K. and Tandon, P. 1986. Influence of growth regulator on asymbiotic germination and early seedling development of *Coelogyne punctulata* Lindl. In: (ed. Vij, S. P.) *Biology, Conservation and Culture of Orchids*. Affiliated East-West Press, New Delhi. Pp. 441-451.

Sharma, S. K. and Tandon, P. 1990. Asymbiotic germination and seedling growth of *Cymbidium elegans* Lindl. and *Coelogyne punctulata* Lindl. as influenced by different carbon sources. *J Orchid Soc India*, 12: 83-87.

Shiau, Y. J.; Nalawade, S. M.; Hsia, C. N.; Mulabagal, V. and Tsay, H. S. 2005. *In vitro* propagation of the Chinese medicinal plant, *Dendrobium candidum* Wall. Ex Lindl., from axenic nodal segments. *In Vitro Cell Dev Biol Pl*, 41: 666-670.

Showkat, R. M.; Mohammed, A. and Kappoor, R. 2004. Chemical composition of essential oil of *Cinnamomum tamala* Nees and Eberm. leaves. *Flavour Fragrance J*, 19: 112-114.

Singh, J. S. and Singh, S. P. 1992. Forest of Himalaya: structure, functioning and impact of man. Gyanodaya Prakashan, Nainital, India.

Sood, A. and Vij, S. P. 1986. *In vitro* root segment culture of *Rhynchostylis retusa* Bl. In: (ed. Vij, S. P.) *Biology, Conservation and Culture of Orchids*. Affiliated East-West Press, New Delhi, India. Pp. 463-468.

Sridhar, T. M. and Naidu, C. V. 2011. *In vitro* Direct Shoot Organogenesis from Leaf Explants of *Solanum nigrum* (L.) - An important Antiulcer Medicinal Plant. *J Phytology*, 3(5): 29-35.

Stals, H. and Inze, D. 2001. When plant cells decide to divide. *Trends Pl Sci*, 6: 359-364.

Sungkumlong and Deb, C. R. 2008. Effects of different factors on immature embryo culture, PLBs differentiation and rapid mass multiplication of *Coelogyne suaveolens* (Lindl.) Hook. *Indian J Exp Biol*, 46: 243-248.

Sungkumlong and Deb, C. R. 2009. Regeneration competence of *Taenia latifolia* (Lindl.) Benth. Ex. Hook. pseudobulb segments: A study *in vitro*. *Indian J Biotech*, 8: 121-126.

Swamy, M. K.; Sudipta, K. M.; Balasubramanya, S. and Anuradha, M. 2010. Effect of different carbon sources on *in vitro* morphogenetic response of Patchouli (*Pogostemon cablin* Benth.). *J Phytol*, 2(8): 11-17.

Temjensangba and Deb, C. R. 2005a. Regeneration and Mass Multiplication of *Arachnis labrosa* (Lindl.ex Paxt.) Reichb: A Rare and Threatened Orchid. *Curr Sci*, 88(12): 1966-1969.

Temjensangba and Deb, C. R. 2005b. Regeneration of plantlets from *in vitro* raised leaf explants of *Cleisostoma racemiferum* Lindl. *Indian J Exp Biol*, 43: 377-381.

Temjensangba and Deb, C. R. 2005c. Factors Regulating Non-Symbiotic Seed Germination of Some Rare Orchids of Nagaland. *Nagaland Univ Res J*, 3: 48-54.

Temjensangba and Deb, C. R. 2006. Effect of different factors on non-symbiotic seed germination, formation of protocorm-like bodies and plantlet morphology of *Cleisostoma racemeferum* (Lindl.) Garay. *Indian J Biotech,* 5: 223-228.

Tiwari, V.; Deo, S. B. and Nath, T. K. 1998. Shoot regeneration and somatic embryogenesis from different explants of Brahmi [*Bacopa monniera* (L.) Wettst.]. *Pl Cell Rep*, 17: 538-543.

Tormmasi, F.; Paciolla, C.; de Pinto, M. C. and de Gara, L. 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Pl Physiol Biochem*, 44: 359-368.

Tyagi, P.; Khanduja, S. and Kothari, S. L. 2005. Somatic embryogenesis in *Capparis deciduas* (Forsk) Edgew – A multipurpose Agroforestry plant. *J Pl Biochem Biotech,* 14: 197-200.

Vacin, E. F. and Went, F. W. 1949. Some *p*H changes in nutrient solutions. *Bot Gaz*, 110: 605-613.

Vaidya, B. G. 1971. Some controversial drugs of Indian medicine II. *J Res Indian Med*, 6(1): 95-104.

Valmayor, H. L. and Sagawa, Y. 1967. Ovule culture in some orchids. *Amez Orchid Soc Bull,* 36: 766-769.

Ved, D. K.; Kinhal, G. A.; Ravikumar, K.; Ghate, U.; Sankar, R. V. and Indresha, J. H. 2003. Conservation assessment and management prioritization for the medicinal plants of

Himachal Pradesh, Jammu & Kashmir and Uttaranchal. In: *Proc workshop*, Shimla, India, May 19-24.

Vij, S. P. 1993. Regeneration response of orchid roots: A study *in vitro*. *J Orchid Soc India*, 7: 61-73.

Vij, S. P. 2002. Orchids and tissue culture: Current status. In: (eds. Nandi, S. K.; Palni, L. M. S. and Kumar, A.) *Role of Plant Tissue Culture in Biodiversity Conservation and Economic Development*. Nainital: Gyanodaya Prakash. Pp. 491-502.

Vij, S. P. and Pathak, P. 1988. Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon*: A study *in vitro. J Orchid Soc India,* 4: 69-88.

Vij, S. P. and Pathak, P. 1990. Micropropagation of orchids through leaf segments. *J Orchid Soc India*, 4: 69-88.

Vij, S. P. and Sharma, M. 1997. A Micropropagation system for Vanda Kasem's Delight Tom Boykin. In: *Proc. Natl. Seminar on Development Biology and Commercialisation of Orchids and Orchid Show*. Gangtok, Sikkim, India. Pp. 85.

Vij, S. P.; Sood, A. and Pathak, P. 1989. On the utility of rhizome segments in micropropagating *Eulophia hormusjii* Duth. *J Orchid Soc India*, 3: 41- 45.

Vij, S. P.; Pathak, P. and Mahant, K. C. 1995. Green pod culture of a therapeutically important species *Dactylorhiza hatagirea* (D. Don) Soo. *J Orchid Soc India*, 9: 7-12.

Walters, C. 2004. Appendix 2 in *Ex situ* conservation, supporting species survival in the wild. Guerrant, Havens and Maunder, Island Press.

Yam, T. W. and Weatherhead, M. A. 1988. Germination and seedling development of some Hong Kong orchids. *Lindleya*, 3: 156-160.

Yapo, E. S.; Kouakou, T. H.; Kone, M.; Kouadio, J. Y.; Kouame, P. and Merillon, J. M. 2011. Regeneration of Pineapple (*Ananas comosus* L.) plant through somatic embryogenesis. *J Pl Biochem Biotech*, 20(2): 196-204.

Yarra, R.; Aileni, M.; Vemunoori, A. K.; Kokkirala, V. R.; Umate, P. and Abbagani, S. 2010. Direct Shoot Regeneration from Mature Leaf Explants of *Sphaeranthus indicus* L., A multipurpose Medicinal plant. *J Phytology*, 2(5): 05-11.

Yasugi, S. 1984. Shortening the period from pollination to getting seedlings by ovule or ovary culture in *Doritis pulcherima* (Orchidaceae). *J Japan Soc Hortic Sci*, 53: 52-58.

****

Plate 1 : Temperate forest of the study area.

Plate 2 : Burning down of forest for shifting cultivation.

Plate 3 : Terrace Cultivation

Plate 4 : Jhum Cultivation


A


B


C


D


E


F

Plate 5 : A. Traditional house of *Tangkhul* tribe,
B. Inside view of the kitchen,
C. Bamboo containers for preserving fermented food materials,
D. Store house of fire woods,
E. Chutney grinder,
F. Wooden plate with stand *(Seichang Khongphei)*.

Plate 6 : A. Traditional palanquin made of *Toona* woods used for carrying dignitaries during auspicious ceremonies, B. Village chief performing the ritual ceremony during *Luira* festival, C. Village couple showing the scene of sowing the first seed of the year during *Luira* festival, D. *La Khanganui* (Virgin's dance), E. *Rai pheichak* (War dance), F. Interview with the village elders.


A


B


C


D


E


F

Plate 7 : A. *Tangkhul* couple attires traditional dress & ornaments,
B. Upper garment for both sexes *(Raivat Kachon)*,
C. Upper garment for male *(Chongkhom)*,
D. Lower garments for female *(Kashon)*,
E. Weaving in a loin loom,
F. Interview with handloom weavers.

A

B

C

D

E

F

Plate 8 : A-D. *Tangkhul* Ornaments,
E. *Tangkhul* youths in traditional dress,
F. *Tangkhul* headgear.

Plate 9 : Edible Underground parts.

A. ***Alpinia galanga*** **Willd.,** **B.** ***Colocasia esculenta*** **(L.) Schott.,**
C. ***Dioscorea alata*** **L.,** **D.** ***Hedychium coronarium*** **Koening,**
E. ***Manihot esculenta*** **Crantz.,** **F.** ***Sechium edule*** **Sw.**

Plate 10 : Leafy Vegetables.

A. *Antidesma acidum* Retz., **B.** *Elatostema lineolatum* Wight.,
C. *Ficus tsjakela* Burm.f., **D.** *Persicaria perfoliatum* L.,
E. *Polygonatum cirrhifolium* Royle, **F.** *Viola pilosa* Blume


A


B


C


D


E


F

Plate 11 : Leafy Spices

A. ***Allium hookeri*** **Thw.,** **B.** ***Allium chinense*** **G.Don.,**
C. ***Allium tuberosum*** **Roxb.,** **D.** ***Apium graveolens*** **L.,**
E. ***Elsholtzia blanda*** **Benth.,** **F.** ***Eryngium foetidum*** **L.**

Plate 12 : Edible Flowers/Inflorescences

A. ***Bauhinia purpurea*** **L.,**

B. ***Clerodendrum farinosum*** **(Roxb.) Steud.,**

C. ***Cucurma angustifolia*** **Roxb.,**

D. ***Phlogacanthus thyrsiformis*** **(Roxb. ex Hardw.) Mabb.,**

E. ***Rhododendron arboreum*** **Sims.,**

F. ***Strobilanthes auriculatus*** **Nees.**

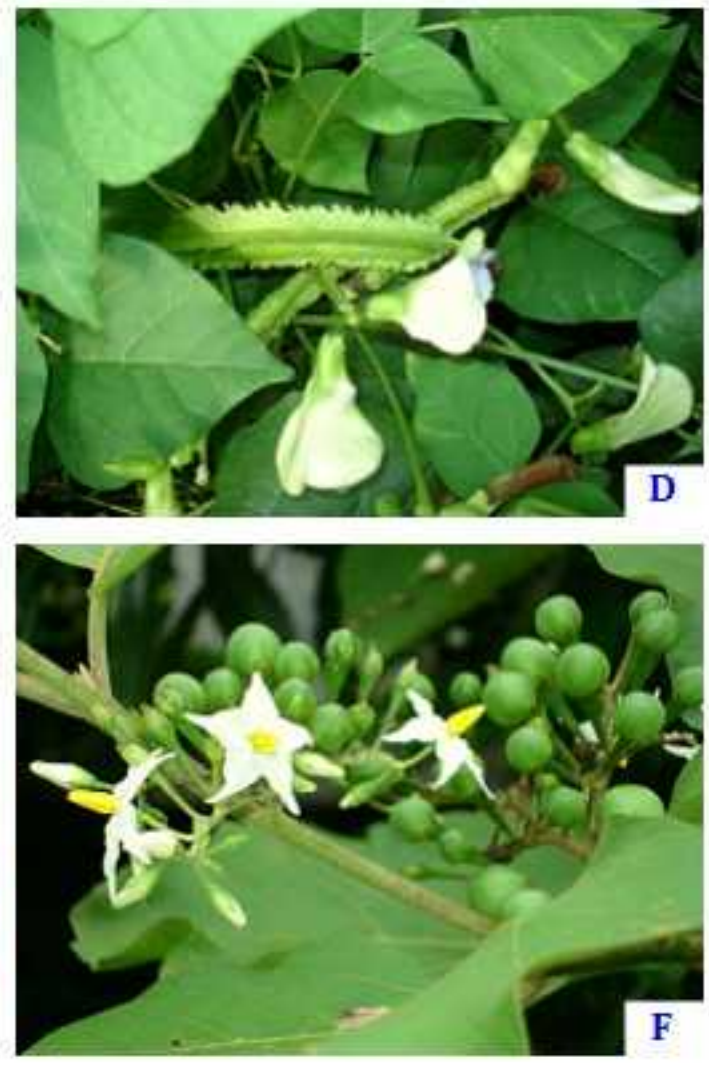

Plate 13 : Fruit Used As Vegetables

A. ***Capsicum chinense*** **Jacq.,** **B.** ***Cyphomandra betacea*** **Cav.,**
C. ***Parkia timoriana*** **Merr.,** **D.** ***Psophocarpus tetragonolobus*** **DC.,**
E. ***Solanum spirale*** **Roxb.,** **F.** ***Solanum torvum*** **Schltdl.**

Plate 14 : Edible Fruits

A. ***Citrus medica*** **L.,** **B.** ***Docynia indica*** **(Colebr.) Decne.,**
C. ***Myrica esculenta*** **Ham.,** **D.** ***Prunus salicina*** **Lindl.,**
E. ***Rhus semialata*** **Murr.,** **F.** ***Viburnum foetidum*** **Wall.**

Plate 15 : Edible Fruits

A. ***Calamus tenuis*** **Roxb.,** **B.** ***Citrus maxima*** **(Burn.) Merr.,**
C. ***Garcinia pedunculata*** **Roxb.,** **D.** ***Prunus nepalensis*** **Steud.,**
E. ***Tamarindus indica*** **L.,** **F. Fruit beer display for sale**

Plate 16 : Edible Plant Parts Display For Sale

A. Shoot of *Arundinaria callosa* Munro, **B.** Young shoot of *Calamus* sp,
C. Fruit of *Hibiscus sadbariffa* L., **D.** Fruit of *Leucana glauca* Benth.,
E. Spadix of *Musa* spp, **F.** Pod of *Vicia faba* L.

Plate 17 : View Of Makeshift Market In Different Localities Of Ukhrul District

A. Selling of leafy vegetables,
B. Selling of *Esholtzia communis* (Coll. & Hemsl.) Diels,
C. Selling of leafy spices & vegetables,
D. Selling of *Colocasia* sp,
E. Selling of tender bamboo shoot,
F. Selling of *Euryale ferox* Salisb., fruits of *Oroxylum indicum* Vent.

A

B

C

D

E

F

Plate 18 : Edible Fungi

A. ***Lactarius princeps*** **Berk.,**

B. ***Lentinula edodes*** **(Berk.) Pegler,**

C. ***Ramaria sanguine*** **(Pers.) Quel.,**

D. ***Termitomyces clypeatus*** **Heim.,**

E. ***Termitomyces eurrhizus*** **(Berk.) Heim.,**

F. Interview with woman vendor in ***Lambui*** **market.**

Plate 19 : Ethnomedicinal Plants

A. ***Debregeasia longifolia*** **(Burm.f.) Wedd.,** **B.** ***Dendrobium denudans*** **D. Don,**
C. ***Pogostemon benghalensis*** **kuntze,** **D.** ***Scutelleria discolor*** **Colebr.,**
E. ***Swertia chirata*** **Buch.Ham.**

Plate 20 : Ethnomedicinal Plants

A. ***Platycodon grandiflorum*** **(Jacq.) A. DC.,** **B.** ***Pratia nummularia*** **Benth.,**
C. ***Stephania hernandifolia*** **(Willd.)Walp.,** **D.** ***Tacca laevis*** **Roxb.,**
E. ***Taxus baccata*** **L.,** **F.** ***Thalictrum foliolosum*** **DC.**

Plate 21 : Ethnomedicinal Plants

A. *Clerodendrum colebrookianum* **Walp.,** **B.** *Entada pursaetha* **DC.,**
C. *Hibiscus sabdariffa* **L.,** **D.** *Homskioldia sanguine* **Retz.,**
E. *Paris polyphylla* **Sm.,** **F.** *Pavetta indica* **L.**


A


B


C


D


E


F

Plate 22 : Ethnomedicinal Plants

A. Woman collecting *Crassocephalum crepidiodes* S. Moore for medicinal uses,

B. *Tangkhul* man showing medicinal plant *Pouzolzia viminea* Wedd.

C. Herbal medicine kept ready for sale,

D-F. Interview with different healers during the study.


A


B


C


D


E


F

Plate 23 : Dye Yielding Plants

A. ***Bixa orellana*** **L.,**
B. ***Mallotus philippensis*** **(Lam.) Mull.,**
C. ***Isodon hispidus*** **Benth.,**
D. ***Mahonia manipurensis*** **Takeda.,**
E. ***Pasania pachyphylla*** **Schot.,**
F. ***Strobilanthus cusia*** **Kuntze**

Plate 24 : Fish Poisoning Plants

A. ***Dillenia indica*** **L.,**

B. ***Engelhardtia spicata*** **Blume,**

C. ***Juglans regia*** **L.,**

D. ***Millettia pachycarpa*** **Benth.,**

E. A man showing stem of ***Acacia pinnata*** **Dalzell & A. Gibson,**

F. Girls practicing fish poisoning by using plant parts of ***Millettia pachycarpa*** **Benth.**

Plate 25 : Plants Used For Hair-Care

A. *Artemisia nilagarica* **(C.B. Clarke) Pamp.,** **B.** *Boehmeria sidaefolia* **Wedd.,**
C. *Cymbopogon citratus* **Stapf,** **D.** *Phyllanthus emblica* **L.,**
E. *Perilla frutescens* **Britton,** **F.** *Sapindus emarginatus* **Vahl.**

Plate 26 : Different Carrying Baskets And Household Equipments Made Of Bamboo And Cane

A. ***Kharing*** **(Transporting basket),** **B.** ***Lungkai*** **(Transporting basket),**
C. ***Bangrah*** **(Transporting basket),** **D.** ***Changuei*** **(Traditional shield),**
E. ***Tabu*** **(Clothe keeping basket),** **F.** ***Chum*** **(For storing** ***Oryza sativa*** **L.)**

Plate 27 : Different Household Equipments Made Of Bamboo And Cane

A. ***Luk*** **(Container for keeping grains),** **B.** ***Kamu*** **(Basket for storing dry fish),**
C. ***Tebam*** **(Basket for keeping thread),** **D.** ***Paipek*** **(Basket for keeping caught fish),**
E. ***Yamkok*** **(Winnowing plate),** **F.** ***Liphang*** **(Traditional dinning table)**


A


B


C


D


E


F

Plate 28 : A. Traditional helmet (*Luiho pasi*),
B. Bamboo basket (For keeping dry fish for sale),
C. Bamboo mug (Made from *Dendrocalamus giganteus* Munro),
D. An old man knitting cane basket,
E. An old woman knitting bamboo basket,
F. *Tangkhul* lady carrying grains in *Kharing & Luk*

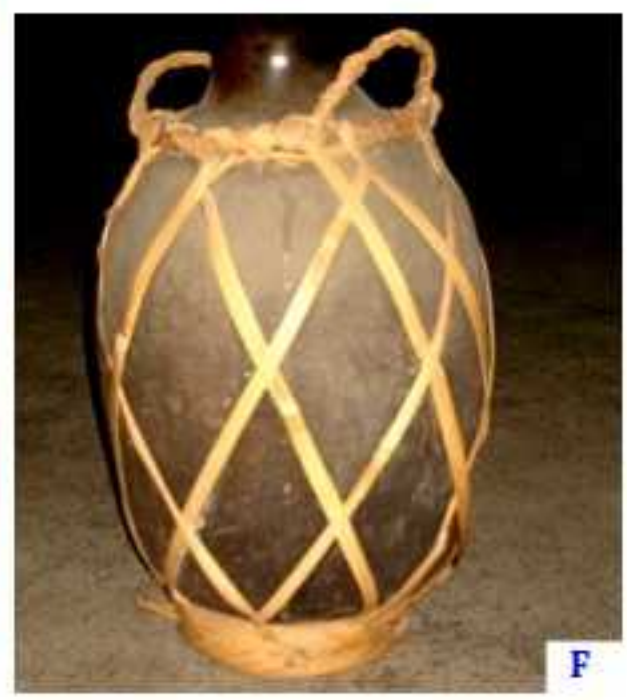

Plate 29 : A-D. Diffrent shapes and sizes of *Lagenaria seciraria* (Molina) Standl. spp. for domestic uses, E. Drinking rice beer from *Khorshon* (Gourd rice beer pot), F. Traditional rice beer pot

Plate 30 : A. ***Tingteila*** **(Traditional stringed instrument),**
B. Different agricultural implements,
C. Decoration items made from bamboo sp
D-E. Decoration items made from ***Gmelina arborea*** **Roxb.,**
F. Flower vase made from ***Dendrocalamus giganteus*** **Munro**

Plate 31 : Plants Of Miscellanous Uses

A. *Aconitum nagarum* stapf .,
B. *Coix lacryma-jobi* L.,
C. *Curcuma caesia* Roxb.
D. *Averrhoa carambola* L.
E. *Duabanga grandiflora* Walp.,
F. *Phrynium capitatum* Wild.