"Clonal Mass Multiplication of Two Economically Important Plants of Nagaland- An *in vitro* Approach"

BY

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DECLARATION

I, T. Arenmongla, bearing Ph. D. registration number 360/2009 (December 06, 2007), hereby, declare that the subject matter of my thesis entitled "CLONAL MASS MULTIPLICATION OF TWO ECONOMICALLY IMPORTANT PLANTS OF NAGALAND - AN *IN VITRO* APPROACH" 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.

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Chapter-1

Introduction

Men have always been fascinated by the diversity of life. Biodiversity is the result 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. The 34 biodiversity hotspots by region are: 4 in North and Central America (*California floristic province, Caribbean Island, Madrean pine-oak woodlands, Meso-America*); 5 in South America (*Atlantic Forest, Cerrado, Chilean Winter Rainfall-Valdivian Forest, Tumbes-Choco-Magdalena, Tropical Andes*); 4 in Europe and Central Asia (*Caucasus, Irano-Anatolian, Mediterranean, Mountains of Central Asia*); 8 in Africa (*Cape Floristic Region, Coastal forest of eastern Africa, Eastern Afromontane, Guinean Forest of West Africa, Horn of Africa, Madagascar and the Indian Ocean Islands, Maputaland-Pondoland-Albany, Succulent Karoo*); 13 in Asia-Pacific (*East Melanesian Island, Himalaya, Indo-Burma, Japan, Mountains of Southwest China, New Caledonia, New Zealand, Philippines, Polynesia-Micronesia, Southwest Australia, Sundaland, Wallacea, Western Ghats and Sri Lanka*). 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.

North East India lies between 21°34'N to 29°50'N latitude and 87°32'E to 97°52'E longitudes and covers an area of ca 262060 sq km. The North-East India is part of both Himalaya as well as Indo-Burma biodiversity hotspots in the world. It forms a unique biogeographic province encompassing major biomes recognized in the world. It has the richest reservoir of plant diversity in India and is one of the biodiversity hotspots' of the world supporting about 50% of India's biodiversity (Mao and Hynniewta, 2000). Takhtajan (1969) named the North-East region "*The cradle of flowering plants*" which is one of the richest and most interesting floristic regions of India. The North-Eastern region of India is the fountain of rich diversity of many economic important plants and also the richest reservoir of genetic variability. North-East India is blessed with almost all types of vegetation and it is estimated that out of 1229 species of orchids known from India, about 750 to 800 species are found in North-East region of the country (King and Pantling, 1898; Pradhan, 1979; Kataki, 1986; Kumar and Manilal, 1994; Chowdhery, 1998; Hynniewta *et. al.*, 2000; Deb *et. al.*, 2003; Deb and Imchen, 2008).

In Nagaland, the angiosperm flora is represented by over 2500 species belonging to near 963 genera and 186 families. Nagaland state lies between 25°6′ - 27°4′ of latitudes and 93°20′ - 95°15′E longitude with geographical area of about 16, 57,900 hectares and forest covers and area of 8, 62,930 hectares. It harbors a large number of plant species which are endemic to the state or the North Eastern part of India. Though most of the species are of

economic importance, some of the species plays a vital role in rural economy and used as medicine, vegetables, construction materials, as dye etc Some of the important economically plant species of Nagaland are: *Acorus calamas, Adiantum philipinense, Aloe vera, Crateva religiosa, Dioscorea* sps., *Distemon indicum, Euginia jambula, Hedyotes scandens, Hypoxis aurea, Lassia spinosa, Livistona jenkinsiana,* ***Malaxis acuminata,*** *Panax pseudoginseng, Paederia foetida,* ***Strobilanthes flaccidifolious,*** *Taxus bacata, Trichopus zeylanicus* and many more. But the population of these economically important plant species are under seize in their natural habitat due to over-exploitation, removal of forest cover for 'Jhum Cultivation', unplanned developmental activities. Amongst the different economically important species some of them play a vital role in rural economy.

Nature has provided a rich storehouse of herbal remedies to cure all mankind's ill. Through out the world, people have utilized several thousands of plants and plant products as cure for human ailments. But there are only few plants which are cultivated for commercial purpose. Of the different medicinal plant used in modern medicine about 50% of these plants are still collected from the wild exclusively.

In India, the ayurvedic system of medicine has been in use for more than three thousand years. **Charaka and Susruta**, two of the earliest Indian authors had sufficient knowledge of the properties of the Indian medicinal plants. ***Charaka Samhita*** and the ***Susruta Samhita*** which are their medical works are one of the esteemed treasures of literature on indigenous medicine today (Deb *et. al.*, 2009)

Orchids are one of the largest and most diverse groups among angiosperms and one of the largest families of the flowering plants comprising about 1000 genera and over more than 17,000 species which is widely distributed all over the world (Deb and Imchen, 2008, 2011)

The orchids are known to mankind for several centuries for their beautiful and attractive flowers. In addition to their ornamental value, orchids are also well known for their medicinal value especially in the traditional folk medicine. The orchids exhibit an amazing diversity in shape, size, color of plant body and flowers. The first book on the cultivation of orchids describing species and varieties was probably written in Chinese around 1000 A.D. Orchids have been used in ancient China and India to cure certain ailments and as tonic. Orchid flowers are remarkable not only in their form and beauty or in their pollination mechanism, but also in their setting and even death. Biologically orchids are highly specialized and able to grow on a variety of substratum and environment. According to their habitat, orchids can be broadly classified into following groups

1) Terrestrial- growing in soil
2) Epiphyte- growing on other plants
3) Saprophyte- growing on dead organic matter.

Some of the important species are *Calanthe, Habenaria, Malaxis*, etc (terrestrial orchids). *Dendrobium, Bulbophyllum, Cymbidium, etc.* (epiphytic orchids), *Galeola falconeri, Galeola nudifolia, Epipogium* species *etc.* (saprophytic orchids). The seeds of orchids are exceedingly small in size, varying in length from 0.09 to 0.27 mm consisting of a simple undifferentiated embryo within a transparent integument or seed coat. Most of today's important plants were known to the early agriculturists. Modern man's contribution to agricultural development has been more in terms of yield improvement than in discovering new species with economic potential. The conventional propagation of the species is too slow and unable to overcome the threat of extinction. The seed germination of terrestrial orchid species under natural condition has been considered to be more difficult than that of epiphytic species (Arditti and Ernst,

1984). *In vitro* propagation strategies are a viable alternative for the rapid propagation of orchids. The *in vitro* germination of orchids are influenced by various factors like light condition (Arditti and Ernst, 1984; Deb and Temjensangba, 2006a; Steward and Kane, 2006; Deb and Sungkumlong, 2008, 2009; Pongener and Deb, 2009), developmental stage of green pods/embryos (Deb and Temjensangba, 2006a; Lee *et. al.*, 2007; Pongener and Deb, 2011a), plant growth regulators (PGRs) etc.

In addition to medicinal value of plants, it also provides us with many other useful products such as dyes, tannins, waxes, resins, flavoring, rubber, etc. While in North East forests provides us with fossil fuels we depend on so heavily today. Many plant parts are pigmented, most plants do not yield good dyes because the chemicals responsible for the colors fade or turn muddy over time. Historically, dyes were water-soluble plant extracts and have been used by man since ancient times for the coloring of fabrics/cloth. Plant dye-stuffs were used by primitive man for coloring animal skins and also his own skin during religious festivals and also during war time. A number of natural dyes are very important and are still prized such as indigo, logwood, woad, safflower, saffron and annatts. They are used for dyeing textiles, leather, paints, varnishes, paper and ink and also used for coloring many foods, beverages and medicines. The earliest authentic records of dyeing are found in the tomb paintings of ancient Egypt, depicting every facet of contemporary life. Nearly 450 taxa are known to yield dyes in India alone, of which 50 are considered to be the most important 10 of these are from roots, 4 from barks, 5 from leaves, 7 from flowers, 7 from fruits, 3 from seeds, 8 from wood and 3 from gums and resins. Some well-known dyes are blue indigo from the leaves of *Indigofera tinctoria* L., red dye made from the roots of *Rubia tinctorum* L., yellow from stigmas of the saffron plant (*Crocus sativus* L.) and also from tumeric (*Curcuma*

longa L.). Dyes might have been discovered accidentally, but their use has become so much a part of man's customs that it is difficult to imagine a modern world without dyes. The art of dyeing spread widely as civilization advanced (Krishnamurthy *et. al.*, 2002; Mahanta and Tiwari, 2005).

The inhabitants of North-East region have been dyeing their cloth with natural dyes from time immemorial. Almost every region seems to have discovered indigenous dye-yielding plants, as many plants are capable of yielding dyes. Natural dyes are environment-friendly and it occurs in almost all plant families and produce dyes from almost all parts of the plant, such as root, bark, leaf, fruit, wood, seed, flower, etc. The content or amount of dye present in the plants varies greatly depending on the season as well as age of the plants. Some of the dyes yielding plants are *Bixa orellana, Carthamus tinctorius, Dactylopius coccus, Ficus altissima, Haematoxylon campechianum, Indigofera spp, Isatis tinctoria, Lawsonia inermis, Pinus wallichiana, Phaius tankervilliae,* ***Strobilanthes flaccidifolious*** etc. The indigenous knowledge of using dyes has decreased to a very great extent. It has been found that the traditional knowledge of dye-making is now confined to few surviving older people in Nagaland. Population of the dye yielding plants is decreasing at an alarming rate due to different anthropogenic activities. Therefore, *in vitro* propagation can be used as an effective alternative for conservation and multiplication of this species in order to ensure its existence on the earth.

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

The North-Eastern region of India 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.

Knudson (1922) for the first time demonstrated the possibility of bypassing the fungal requirements during germination of *Cattleya* seeds *in vitro* by culturing the seeds supplemented with appropriate organic carbon in the medium, while Tsuchiya (1954) discussed the possibility of germinating orchid seeds from immature pods. The discovery of these two techniques led to the development of 'green pod culture' that enabled to rescue hybrid embryos from desired mating (Sagawa, 1963). However, it calls for devising protocols for rapid cloning for exploitation of elite hybrids. *In vitro* cloning of *Phalaenopsis* using uninodal floral stock cuttings was developed by Rotor (1949), and Thomale (1957) successfully cultured the shoot tips of *Orchis maculata*, but the possibility of using aerial roots for micropropagation was first suggested by Beechey (1970). Morel (1960) is credited for mass propagation of virus free *Cymbidium* clones from apical shoot meristem on Knudson 'C' medium. Shoot tips remain the most commonly used explants for micropropagating *Cymbidium* species and other sympodial orchids but their utility is limited in monopodials as it involves the removal of the only growing apex, which endangers the survival of the mother stock. Endeavors should, therefore, be made towards exploring an alternative but equally effective technique which excision will not be detrimental to the survival of the mother plant. Different workers have reported regeneration of plantlets in cultures using different explant sources like shoots, roots, seeds, axillary buds, pseudobulbs, leaf (George and Ravishankar, 1997; Sinha and Hegde, 1997; Nayak *et. al.*, 1997; Vij and Pathak, 1999; Prasad *et. al.*, 2000; Vij *et. al.*, 2000; Temjensangba and Deb, 2005a, b, c, 2006; Deb and Temjensangba, 2005, 2007a, b; Abhyankar and Reddy, 2007; Wang X *et. al.*, 2009; Li and Xu, 2009; Deb and

Imchen, 2010; Deb and Sungkumlong, 2010; Dhavala and Rathor, 2010; Hong *et. al.*, 2010; Negi and Saxena, 2011; Sharanappa and Rai, 2011; Swamy *et. al.*, 2010; Devi *et. al.*, 2011; Preethi *et. al.*, 2011) and through callus induction and somatic embryogenesis (Ishii *et. al.*, 1998). Biotechnological tools like plant tissue culture techniques have thus opened new possibilities in conservation of threatened/endangered plants.

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 ex-plants 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, 2006b); *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, 2011b); *C. aloifolium* (seed- Pongener and Deb, 2011a); *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. hornusjii* 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, 2006a); *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 including orchids 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 in orchid is variously referred to as ovule/embryo/green pod/green fruit culture (Sagawa, 1963), which ensures better germination frequency and favors the production of virus free seedlings at a faster rate. Asymbiotic/non-symbiotic seed germination is the most common approach used in the propagation of tropical orchids, which tend to be easier to grow than their temperate relatives. The media used for asymbiotic

germination are more complex than that for symbiotic germination, as all organic and inorganic nutrients and organic carbon source must be in a form readily available to the orchid without the intermediary fungus (Mc Kendrick, 2000). The technique involves an easy procedure for sterilization, ensures better frequency of germination, and reduces the time-lapse between pollination, sowing of seeds and production of virus free seedlings. Since all the seed/embryos are used in a single sowing in this technique, it is important to determine the harvest time of capsule or pod for getting optimal germination. 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. The fruit/capsule

that develops prominent ridges along the valves and ceases to grow in diameter is considered a useful marker for selecting the right stage for embryo culture (Vij, 1995).

Meristem Culture

Resident meristem: The embryo culture produces a great deal of heterozygosity in their progeny in orchids due to its out breeding characteristic. Because of this, it appears to be a disadvantageous proposition in cut-flower industry where pure lines of desired genotypes are preferred. The possibility of using excised shoot-meristem of *cymbidium* species for regenerating complete plant from *in vitro* was first demonstrated by Morel (1960), whereas Wimber (1963) formulated, described and published a procedure for the purpose. This technique of using resident meristem (shoot-tips, axillary bud) has opened new vistas in orchid 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. However, it has limited utility in monopodial taxa as it involves the sacrifice of the growing tip thereby, endangering the survival of the mother plant.

Adventive meristems: The ability to use an adventive meristem is advantageous as it does not endanger the survival of mother plant. The regenerative competence or the proliferative potential has been positively tested in many orchid taxa, viz: leaf explants (Vij *et al.*, 1984; Mathews and Rao, 1985; Chaturvedi and Sharma, 1986; Seeni, 1988; Vij and Pathak, 1988, 1990; Seeni and Latha, 1992; Temjensangba and Deb, 2005b; Deb and Temjensangba, 2007a; Arumugam *et. al.*, 2009; Deb and Sungkumlong, 2010; Ochoa *et. al.*, 2010; Preethi *et. al.*, 2011; Sridhar and Naidu, 2011); root (Chaturvedi and Sharma, 1986; Deb and Temjensangba, 2005, 2006a; Sood and Vij, 1986; Vij, 1993; Vij and Pathak, 1988); flower stalks (Kaur and Vij, 1995; Singh and Prakash, 1984; Vij *et. al.*, 1997a). 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 Protocorm-like-bodies (PLBs). Somatic embryogenesis is either direct or callus mediated development, and multiplication and differentiation of the PLBs is influenced by the chemical stimulus present in the nutrient pool (Seeni and Latha, 1992; Vij and Pathak, 1990).

The advantages of leaf and root segment 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), Mitra *et al* (Mitra *et. al.*, 1976), Murashige and Skoog (MS) (1962), 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 PLBs in *Luisia trichorhiza, Satyrium nepalense, Vanda cristata* and *Vanda testaceae* leaf segment culture (Vij, 1995). 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).

Plant Tissue Culture Media and Substrata

Since its introduction as a gelling agent for microbial cultures more than 100 years ago, agar has been extensively used as gelling agent for microbial as well as plant tissue culture media (Babbar and Jain, 2006, Deb and Pongener, 2010; Pongener and Deb, 2011b). Agar is useful for the purposes due to its stability, high clarity, non-toxic nature and resistance to its metabolism (Babbar and Jain, 2006; Henderson and Kinnersley, 1988; Mc Lachlan, 1985). In the recent past several attempts have been made to look for some suitable substratum which can replace agar in the plant tissue culture media as well as microbial culture because of doubts about its inertness and non-toxic nature, fear of over-exploitation of its sources and above all the exorbitant price of tissue culture and bacteriological grade agar (Kohlenbach and Wernicke, 1978; Debergh, 1983; Arnold and Ericksson, 1984; Singha, 1984; Babbar and Jain, 1998, 2006; Zimmerman *et. al.*, 1995; Jain and Babbar, 2002; Deb and Pongener, 2010). During the last two decades, a number of substances viz. alginates (Scheurich *et. al.*, 1980), agarose (Johansson, 1988), gelrite (Pasqualetto *et. al.*, 1988), isubgol (Babbar and Jain, 1998), starch (Zimmerman *et. al.*, 1995; Nene *et. al.*, 1996), Xanthan gum (Jain and Babbar, 2006) etc. have been used with reasonable success as substitutes of agar. But these are not expected to find universal acceptance, for various reasons. Alginates gel only in the presence of specific ions and therefore are not suitable substitutes of agar, while agarose is cost prohibitive. Starch is not expected to find universal acceptance because of its inferior gelling ability, poor clarity and metabolizable nature, which leads to softening of the media. Isubgol, due to its polysaccharide nature, good gelling ability, resistance to enzymatic

activity, and gel clarity it has a good potential to become a universal gelling agent for plant tissue culture media. But its high melting point (~70°C) necessitates adjusting of *p*H and fast dispensing (Babbar and Jain, 2006). But use of these gelling agents does not help in substantially reducing the production costs.

Objectives of the Present Study

Phytogeographical studies have revealed that North-East India harbors about 50% of the total Indian flora (about 10,000 species). The region is endowed with ideal climatic conditions for the growth of vegetation of diverse types. The forests are a rich source of plants for food, fodder, timber, fuel, medicine and other forest products. A large area of the natural forests has been destroyed due to "Slash and Burn Cultivation" or "Jhum cultivation". On the other hand, for a number of taxa of this region, potentialities are yet to be exploited. Therefore, the preservation of plant genetic resources of promising as well as threatened types for posterity needs top priority. The use of plant tissue culture technique is visualized for the conservation of rare/threatened/endangered species in mass multiplication of the species with reproductive problems and/or having extremely reduced populations. A wide range of economically important, threatened and endangered plants 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 micropropagation technique is costlier in comparison to conventional propagation. To popularize the tissue culture technique for propagating the horticultural and

Figure 1: Selected plant species showing the vegetative parts with flowers. **a**. *Malaxis acuminata* D. Don; **b**. *Strobilanthes flaccidifolious* Nees.

Figure-1

other economically important species it is necessary to reduce the production cost considerably so that the technique is popular and well taken by horticulturist and other commercial scale growers. In tissue culture one of the costliest ingredients is agar. During the present investigation, efforts have been made to investigate into the possibility of using some low cost substrata which could be used as alternative to agar to reduce the production cost.

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.

For the current research work, two economically important plant species viz, ***Malaxis acuminata*** D. Don (Orchidaceae) and ***Strobilanthes flaccidifolious*** Nees (Acanthaceae) have been selected. A brief about the two selected species is given below:

1. *Malaxis acuminata* D. Don (Orchidaceae)

Malaxis acuminata is a small, medium-sized terrestrial orchid, up to 30 cm high, with pseudo bulbs at the base and with fibrous roots, flower pedicellate, yellowish green, purplish near the centre up to 1.5 cm long (**Fig. 1a**). Leaves have sheating leaf base and new plants grow along the vicinity of the decaying mother plant. It grows in shady areas and in forest litter. It usually flowers during the month of June-July. Amongst 19 species of the genus *Malaxis* is present in India; most of them are components of Ayurvedic drug preparations. The dried pseudobulbs are important ingredient of 'Ashtavarga' drugs used in the preparation of Chyvanprash, an energetic herbal tonic, it is also used for providing strength, enhancing sperm formation and to cure tuberculosis, cold and cough. The propagation of orchids is hampered because of low viability of seeds and mandatory mycorrhizal association for seed

germination. Due to over collection for herbal drugs and removal of forest cover the species is under threat.

2. *Strobilanthes flaccidifolious* Nees (Acanthaceae)

It is a glabrous shrub of about 2-3 m high, leaves elliptic acute at both ends. Flowers in densely lax spikes. Bracts petioled, ovate, deciduous. Calyx linear, spathulate, corolla long glabrous. It flowers during the month of December-January, having a light to dark purplish flower (**Fig. 1b**). The leaves are pounded and boiled with water, which produces a light to deep blue color dye and is used for dyeing cloths and fiber by the local handloom industry. Due to over collection of leaves and plant parts the distribution is restricted in few patches in Nagaland. The population of the species is very thin and demand immediate recovery.

In the present investigation for my Ph. D programme, work has been done on the following aspects:

1. **To develop suitable protocols for rapid mass multiplication from different explants sources (seed, leaf, nodal segments, pseudobulb);**
2. **Screening of some low cost substrata as alternative to agar to reduce the production cost;**
3. **Reintroduction of the regenerates in the wild/botanical garden of the University.**

Chapter-2

Malaxis acuminata D. Don

2.1. Materials and Methods

Plant Collection

Malaxis acuminata plants were collected from the forests of Meinkong, Longkhum and Alichen of Mokokchung district, Nagaland. The collected species were maintained in Botanical garden/experimental garden at a temperature of ~20/15°C (day/night). Regular surveys were carried out in the same area at regular intervals to study their status. Though the populations of *Malaxis acuminata* were found in few areas but they were less/absent in Meinkong. Moreover, the size of seed pod of *M. acuminata* is very small which place restrictions on the choice of methods.

Selection of Explants and Sterilization

Seed: Immature seeds/embryos of different developmental ages of 4-12 weeks after pollination (WAP) were harvested at one wk interval from the experimental garden and were used for the present study. The green pods were surface sterilized with a solution of $HgCl_2$ (0.2%, w/v) for 5 min and washed 4-5 times with sterilized distilled water. The pods were dipped in ethanol and flamed just before scoping out the seeds.

Leaf: Leaves were collected from 5-6 wk old plantlets from *in vitro*/axenic culture.

Nodal explants: The nodal explants were collected from the *in vitro* raised etiolated plants. After the plantlets were taken out in the laminar flow cabinet, the leaves were removed and cut into segments with one or two nodes in each segment which were then used to initiate the culture.

Both *in vitro* grown leaves and nodal explants were soaked in sterilized distilled water till used.

Pseudobulb: About 2.0 cm long freshly sprouted pseudobulbs (~3-4 wk after emergence) from polyhouse grown plants were used as explants source. The pseudobulbs collected from *in vivo* source were freed of leaves and scales and scrubbed thoroughly with 'Labolene' (a liquid laboratory detergent, 1:100, v/v ratio) before washing under running tap water and successively sterilized with $HgCl_2$ (0.25%, w/v) (~3 min) followed by ethanol (70%, v/v) (20-30 Sec). The explants were subsequently rinsed 4-5 times with sterilized distilled water after every treatment. Pseudobulbs were also harvested from axenic sourced cultures. Plantlets were taken out inside the laminar flow cabinet and the leaves were removed carefully with the help of forceps. After removing the leaves, the pseudobulbs were cut horizontally into 2 equal halves and the pseudobulb segments were inoculated for culture initiation.

Priming of Explants

After harvesting and sterilization, the leaves, nodal segments and pseudobulbs were soaked in sterilized distilled water till cultured on initiation medium/media.

Preparation of Substrata

Besides agar as gelling agent, three other types of substrata viz., coconut coir, forest leaf litter and polyurethane foam (hereafter called foam) were used as substrata/supporting materials as alternative to agar for regeneration and mass multiplication of plantlets. 'Foam' was collected from the local market which is generally used for preparation of mattresses. While coconut coir was extracted from the dried fruits and forest leaf litters were collected from the forest floor. All the three substrata were soaked with 'labolene' (a commercial laboratory detergent) at 1:100 ratio (v/v) for about two h followed by washing under running tap water till water ran clear. The substrata were air dried and stored till used. The dried coconut coir and forest litter were chopped into small pieces (~0.5 cm size), while foam was cut into disks (according to the size of culture vials). Substrata were then autoclaved at1.05 kg cm^{-2} pressure and 121°C for one hr before putting them in the culture vials.

Tissue Culture Media

For culture of immature seeds/embryos, MS medium (Murashige and Skoog, 1962) and Mitra *et al* (Mitra *et. al.*, 1976) media were used. Both the basal media were fortified with different organic carbon sources such as dextrose, glucose and sucrose (0-4%, w/v) and gelled with agar (0.8%, w/v) (make Hi- media, India) and supplemented with different quality and quantity of plant growth regulators (PGRs) like α-naphthalene acetic acid (NAA) and benzyl adenine (BA) at concentrations of 0-10 μM which were used either singly or in combination.

For initiation of cultures from foliar and nodal explants, the basal media were fortified with sucrose (3%, w/v) as organic carbon source and different concentrations of PGRs like NAA and BA (0-9 µM) either singly or in combination and agar (0.8%, w/v) as gelling agent.

For pseudobulb culture, two basal media viz., MS and Knudson 'C' (Knudson, 1946) were tested; the basal media were fortified with different organic carbon sources like dextrose, glucose and sucrose (0-4%, w/v), CH (100 mg L^{-1}), citric acid (100 mg L^{-1}) as antioxidant and activated charcoal (AC) (0-0.4%, w/v). The culture media was then supplemented with different levels of PGRs like NAA and BA (0-9 µM) either singly or in combination.

The *p*H of the medium 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 cotton plugged. The medium was sterilized by autoclaving at 121°C for 20 min at a pressure of 1.05 kg cm^{-2}. Besides agar, different pre-processed substrata like coconut coir, foam and forest leaf litter were used to which about 12 ml of the liquid media were dispensed in each test tube. The media were autoclaved at 121°C for 20 min at the pressure of 1.05 kg cm^{-2}.

Plant Tissue Culture

Initiation of cultures

Seed: Immature seeds/embryos of various developmental ages were scoped out in the laminar flow cabinet with the help of scalpel and cultured on two basal media containing different organic carbon sources (0-4%, w/v), agar (0.8%, w/v) as gelling agent and supplemented with different concentrations of PGRs such as NAA and BA at different levels (0-10 µM) either singly or in combination. Approximately 200 seeds were sown on the medium in each test tube. For each treatment 20 test tubes were maintained. The cultures were maintained in three

different light conditions viz. at full laboratory illumination (40 µmol m^{-2} s^{-1}), half light illumination (20 µmol m^{-2} s^{-1}) and dark at 12/12 h (Light/Dark) photoperiod and at 25±2°C.

Foliar explants: Young leaves from *in vitro* raised plants were taken out inside the laminar flow cabinet carefully and cultured on MS medium fortified by sucrose (3%), and different levels of PGRs like NAA, BA (0-9 µM) either singly or in combination. For each treatment 20 leaves were used. The foliar explants were cultured in three different orientations (up side up, up side down and horizontal).

Nodal explants: *In vitro* grown plantlets which were etiolated were selected and the cut segments with nodes were cultured for initiation on MS medium containing sucrose (3%) supplemented with different concentrations of PGRs such as NAA and BA (0-9 µM) either singly or in combination and congealed with agar (0.8%, w/v). For each treatment 20 explants were maintained.

Pseudobulb: Pseudobulb segments from both the sources were inoculated on MS and Knudson 'C' media fortified with different organic carbon sources such as dextrose, glucose and sucrose (0-4%), CH (100 mg L^{-1}), citric acid (100 mg L^{-1}) as antioxidant. The media were further fortified with different levels of PGRs like NAA and BA (0-9 µM) singly or in combination. In another set of experiments, AC (0-0.4%, w/v) was added in addition to other adjuncts to study its effect on the morphogenetic response. Pseudobulb segments were cultured on agar-gelled medium as well as 3 other substrata (coconut coir, foam and forest leaflitter).

Experimental design

A completely randomized experimental design was performed. In all the experiments each treatment had at least 5 replicates. Except immature embryos, all other cultures were

maintained at 25±2°C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} and 12/12 each (light/dark) photo period. All the cultures were sub-cultured at 4-5 wk interval unless mentioned otherwise. *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.

The PLBs/shoot buds formed from the germinated embryos/seeds, foliar explants, nodal segments and pseudobulb were maintained for another two passages on optimum initiation condition for further differentiation.

Regeneration of plants and culture proliferation

The advance stage PLBs (PLBs with first set of leaflets) and young plantlets were transferred on two different media namely MS and Mitra *et al* (Mitra *et. al*, 1976) media. The media were supplemented with different organic carbon sources like dextrose, glucose and sucrose (0-4%, w/v) and two different PGRs (NAA and BA) at a concentration of 0-9 µM either singly or in combination. A set of young plantlets were also maintained on medium containing AC (0-0.5%, w/v) along with optimum PGRs to test the effect of AC on culture proliferation and regeneration. All the cultures were maintained at full laboratory illumination.

Apart from agar gelled media, the young tiny plantlets were also maintained on different alternative substrata such as foam, coconut coir and forest leaf litter for regeneration and culture proliferation. The shoot buds/plantlets so formed were separated from the clumps in every sub-culture and transferred on fresh regeneration medium. The plantlets were maintained for 2-3 passages on regeneration medium before transferring them for hardening.

Different strengths of MS medium were also used for plantlet regeneration and mass multiplication. Plantlets of about 1-1.5 cm with 1-2 leaves were cultured on ¼, ½, 3/4 and full strength MS medium with sucrose (3%) and agar (0.8%) with optimized PGRs.

Hardening of regenerates

The well rooted plantlets were taken out from the regeneration medium and transferred on ½ strength MS semi gelled medium containing different substrata (as mentioned above), sucrose (2%, w/v) and devoid of any PGRs and maintained in normal laboratory conditions for 6-7 wk prior to transferring to the potting mix.

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 with the help of a fine brush. The hardened plantlets were then transplanted onto community potting mix (CPM) containing a mixture of charcoal pieces, chopped forest litters, coconut husk, sand and black soil (at 1:1 ratio) with a moss topping.

The CPM were covered with holed transparent polybags and watered at week interval for one month. The plants were fed with $1/10^{th}$ MS salt solution once in a week for 3-4 wk and maintained in polyshade with Ca. 70% of shading sunlight, before transferring them to the wild.

2.2. Results

Initiation of cultures

Immature embryos/seeds

The immature seeds/embryos were cultured on different basal medium containing different supplements. Developmental age of immature embryos, basal media composition,

Table 1: Effect of PGRs on germination of immature embryos of *Malaxis acuminata* *

PGRs Conc. (μM)** NAA	BA	Time taken for initial response (days)	Avg. Time taken for PLBs formation (days)	% germination (±SE)***
0	0	105	170	10 ±1.0[g]
2	0	74	160	75 ±2.5[b]
4	***0***	***55***	***135***	***85*** ±2.0[a]
6	0	58	130	62±3.0[c]
8	0	61	140	62 ±3.0[c]
10	0	85	140	50 ±2.5[e]
0	2	81	160	71 ±1.5[b]
0	4	72	150	66 ±3.0[c]
0	6	89	150	57 ±3.0[d]
0	8	84	145	55 ±2.0[d]
0	10	93	150	50 ±2.5[e]
2	2	73	160	40 ±2.0[f]
2	4	88	155	40 ±3.5[f]
6	4	95	155	62 ±2.0[c]

Data represent the mean of five replicates.

* Immature embryos of 7-8 WAP were cultured on MS medium supplemented with sucrose (3%).

** Only the significant treatments are computed.

*** Standard error. Values followed by the same *letters* are not significantly different from each other.

Table 2: Effects of PGRs on germination of immature embryos of *Malaxis acuminata* on Mitra *et al* medium

PGRs Conc. (µM) NAA	BA	Time taken for initial response (days)	Time taken for PLBs formation (days)	% germination (±SE)*
0	0	155	180	05 (0.5)^{g}
2	0	92	174	53 (1.0)^{b}
4	***0***	***62***	***148***	***65 (1.5)^{a}***
6	0	69	146	50 (2.5)^{b}
8	0	71	165	48 (1.5)^{c}
10	0	88	157	35 (2.5)^{e}
0	2	91	170	50 (1.0)^{b}
0	4	82	159	55 (2.0)b
0	6	102	174	40 (1.0)^{d}
0	8	114	155	35 (2.5)^{e}
0	10	110	155	25 (0.5)^{f}
2	2	85	170	30 (1.0)^{e}
2	4	95	165	35 (1.0)^{e}
6	4	110	175	45 (3.0)^{c}

* Standard error, Values followed by the same *letters* are not significantly different from each other.

Immature embryos of 7-8 WAP were cultured on medium with sucrose (3%)

Data represents the mean of 5 replicates each

Only significant treatments are computed.

Figure 2: Asymbiotic immature seed/embryo germination of *Malaxis acuminata*. **a.** Immature seed/embryo showing nodular swelling; **b.** PLBs and advanced stage PLBs formed from the germinated embryos; c. Regeneration of plantlets and multiple shoot bud formation on regeneration medium.

Figure - 2

quantity of organic carbon, quality and quantity of PGRs were found to be crucial factors for successful culture initiation. Nodular swelling of seeds was the first sign of germination.

Effect of green pod age. In the present study, asymbiotic germination of immature embryos/seeds largely depended on developmental stage of immature embryos at which they were harvested and cultured. Seeds of 7-8 WAP exhibited germination of ~85% after 135 days (19 wk) of culture initiation. Nodular swelling of embryos/seeds followed by bursting out from testa was observed after 8 wk of culture (**Fig. 2a**). Seeds younger than 6 WAP either did not germinate or exhibited delayed germination while, seed age >9 WAP, germination frequency was comparatively lower (data not presented).

Effects of basal media on asymbiotic seed germination. First sign of germination was observed as nodular swelling of the embryos after 55 days of culture followed by healthy and green PLBs formation after 135 days of culture (**Table 1**, **Fig. 2a, 2b**). Amongst the two basal media used for asymbiotic germination of seeds in the present study, MS medium was found to be superior over Mitra *et al* medium (**Table 1, 2**). Under optimum condition germination rate of ~85% was achieved on MS medium against 65% on Mitra *et al* medium (**Table 2**). Embryos cultured on MS medium supported into green and healthy protocorm like bodies (PLBs) formation while, cultures maintained on Mitra *et al* medium resulted smaller PLBs.

Effects of different organic carbon sources and other adjuncts on asymbiotic culture of embryos. Different concentrations of various organic carbon sources viz., dextrose, glucose and sucrose (0-4%, w/v) were also incorporated in the germination media. Incorporation of one of the organic carbon in the initiation medium was prerequisite for successful germination of seeds/embryos. Only nodular swelling of embryos was observed on medium devoid of any organic carbon but no PLBs developed. Amongst the different quality and quantity of organic

Table 3: Effects of different organic carbon sources on germination of immature embryos/seed of *Malaxis acuminata*

Organic carbon source & Conc.(%)	Avg. time taken to germinate (days)	Type of response*
0	115	Few PLBs formed.
Dextrose		
1	71	Few small green PLBs formed.
2	80	As above.
3	75	Nodular swelling with white hairy structures observed.
4	78	As above.
Glucose		
1	89	Very few PLBs are formed.
2	75	As above
3	73	Seed swelled but few green PLBs formed.
4	85	Delayed germination and few healthy PLBs formed
Sucrose		
1	62	Few small green PLBs developed.
2	68	Moderate germination but few green PLBs formed.
3	55	**Embryos germinated well followed by PLBs formation and few plantlets**
4	75	Moderate germination but few green PLBs formed

* On MS medium containing NAA (4 μM) and seed pod of 7-8 WAP.
Data represents the mean of five replicates.

Table 4: Effect of different light intensities on asymbiotic embryo germination of *Malaxis acuminata*

Light conditions	Time taken for Initial response	Time taken for PLBs formation	% Germination (±SE)*	Type of response**
Dark	38	170	70±2.0^{c}	Embryos germinated, round hairy structures formed
Diffused light (20 µmolm^{-2}s^{-1})	50	148	72±2.0^{c}	Nodular swelling with white hairy structures observed, few converted to healthy PLBs
Full light (40µmolm^{-2}s^{-1})	55	135	85±2.0^{a}	Embryos germinated well followed by PLBs formation and few plantlets

* Standard error, Values followed by the same *letters* are not significantly different from each other.

** On MS medium with sucrose (3%) and NAA (4 µM)

Data represents the mean of five replicates each.

Figure 3: Different stages of *in vitro* morphogenetic response of foliar explant of *Malaxis acuminata*. **a**. Cultured leaf explant; **b**. Cultured leaf explant swelling at the basal part and formation of shoot buds; c. Plantlets/multiple shoot buds formed from the cultured leaf.

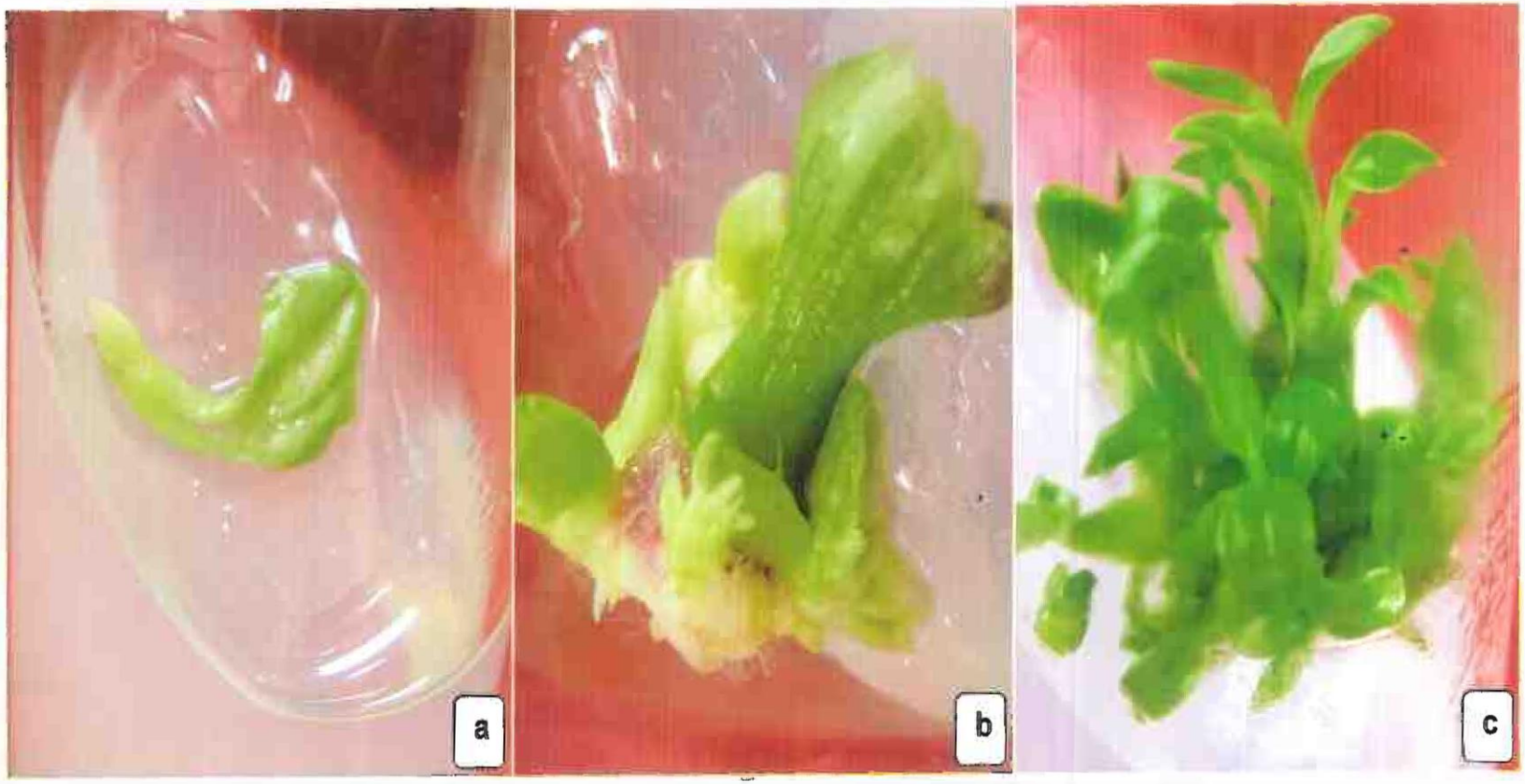

Figure - 3

carbons used, sucrose at a concentration of 3% supported optimum germination and seeds germinated early (**Table 3**). At lower concentration of sucrose, fewer PLBs were formed while at higher concentration, there was delayed germination. Both dextrose and glucose could not support healthy germination.

Effects of quality and quantity of PGRs on asymbiotic germination of embryos: Amongst the different levels of PGRs used for non-symbiotic seed germination, the MS medium containing NAA (4 µM) singly supported optimum germination (~85%) after 55 days of culture and formation of healthy PLBs after 135 days of culture. At lower concentrations of NAA germination delayed while at higher concentrations germination rate was poorer (**Table 1**). The lone treatment of BA across the concentrations and in combination with NAA delayed germination and formed fewer PLBs.

Effect of light on in vitro culture of immature embryos: In the present study, three different light conditions were tested for *in vitro* seed germination. Nodular swelling of embryos was observed within 38 days in the dark and within 55 days a similar response was registered under full laboratory illumination (i.e., 40 µmol m^{-2} s^{-1} light intensity) **(Table 4)**. Though nodular swelling of seeds was faster in the dark, optimum germination was registered with cultures maintained under full light conditions (40 µmol m^{-2} s^{-1}). While, cultures maintained in the dark delayed germination. And cultures maintained in diffused light supported moderate germination.

Foliar explants

Cultures were also initiated from the foliar explants of ~5-6 wk old from *in vitro* source (**Fig. 3a**). Direct shoot buds/PLBs were induced from the basal part of the foliar explants. The foliar explants were cultured in different orientations with the objective to study

Table 5: Effect of orientation of leaf of *Malaxis acuminata* on morphogenetic response

Orientation ofleaf	No. of shoots formed	% response (±SE)*	Average time taken to response	Type of response**
Upside up	18	75 (2.0)[a]	38	Hairy structures, shoots small, developed from petiole
Upside down	12	50 (2.5)[c]	46	Leaf curled, shoots small, unhealthy, degenerated gradually
Horizontal	26	65 (2.0)[b]	28	Shoots developed from the petiole, shoots healthy, hairy structures, shoots formed in clusters, healthy

* Standard error, Values followed by the same *letters* are not significantly different from each other.

** On MS medium with sucrose (3%) and NAA+ BA (3+6 µM in combination)

Data represents the mean of five replicates.

Table 6: Effects of PGRs on morphogenetic response of foliar explants (5 wk old) of *M. acuminata* from *in vitro* source

PGRs Conc. (µM) NAA	BA	Time for initial response (days)	% response (±SE)*	No. of shoots formed	Type of response**
0	0	-	-	-	No response
0	3	35	63 (1.0)[a]	25	Leaf curled followed by hairy structures, shoots formed
0	6	47	30 (1.5)[e]	3	Curling of leaf, shoots developed from vein
0	9	52	45 (2.5)[d]	6	Leaf curled, shoots developed from petiole, white in color. Degenerated gradually
3	0	46	55 (2.5)[c]	15	Hairy structure. Few healthy plantlets developed, shoots small
6	0	-	-	0	No response
9	0	-	-	0	As above
3	3	31	60 (1.7)[b]	21	Swelling at petiole, healthy shoots developed from petiole. Roots developed,
3	*6*	*28*	*65* (1.5)[a]	*26*	***Shoots healthy, hairy structures, shoots formed in clusters, healthy plantlets***
3	9	34	60 (2.0)[b]	14	Shoots small, round structures, degenerated gradually
6	3	42	55 (2.0)[c]	8	Hairy structures, shoots small, developed from petiole
6	6	55	25 (1.0)[f]	0	Slight swelling, but gradually degenerated
6	9	55	45 (3.0)[d]	10	Shoots small
9	3	-	-	0	No response
9	6	60	30 (2.0)[e]	3	Leaf curled, shoots small, unhealthy
9	9	-	-	0	No response

* Standard error, Values followed by the same *letters* are not significantly different from each other.

** On MS medium with sucrose (3%)

Data represents the mean of 5 replicates

Data collected after 50 days of culture

Figure 4: Different stages of *in vitro* morphogenetic response of nodal explants of *Malaxis acuminata*. **a**. Cultured nodal explants; **b**. Sprouting of new shoot buds from the cultured nodal explants; **c**. Multiple shoot buds/PLBs formed from the cultured nodal explants.

Figure - 4

the effect of explants orientation on morphogenetic response. Of the different orientations, the explants placed horizontally on culture medium exhibited better morphogenetic response in comparison to other two orientations i.e., up side up and reverse orientation (**Table 5**). After 4-5 wk of culture initiation of meristematic activity was invoked at the basal ends of the explants. Mostly the meristematic activity was restricted towards the lower basal parts (**Fig. 3b**). In general, of the various PGRs tested in different concentrations for the initiation of culture, the single treatment of NAA and BA did not support healthy morphogenetic response. Single treatment with NAA did not support healthy organogenesis and in most of the cases explants callused while, lone treatment with BA supported moderate organogenesis. But optimum morphogenetic response was recorded on basal medium enriched with NAA (3 µM) and BA (6 µM) in combination (**Table 6**). About 65% of cultured explants responded positively after 28 days of culture initiation on MS medium containing sucrose (3%) and NAA + BA (3 and 6 µM respectively in combination) where as many as 26 meristemoids/shoot buds invoked and formed shoot buds/PLBs (**Table 6, Fig. 3** c). But at higher concentrations morphogenetic response declined significantly and in many cases explants degenerated.

Nodal explants

Cultures were also initiated witlt nodal segments from *in vitro* source (**Fig. 4a**). Sprouting of the shoot buds/PLBs from the nodal region of the segments was initiated witltin 3 wk of culture (**Fig. 4b**). When used singly, NAA proved to be inferior against BA. Medium enriched with NAA supported fewer shoot buds/PLBs formation while, BA enriched medium supported moderate response. But optimum number of shoot buds/PLBs formation and per cent response was registered on medium fortified with NAA and BA in combinations. As

Table 7: Effects of PGRs on morphogenetic response of nodal explants of *M. acuminata* from *in vitro* source

PGRs Conc. (µM) NAA	BA	Time for initial response (days)	No. of shoots formed (±SE)*	No. of roots formed (±SE)*	Type of response**
0	3	30	8^{c} (1.0)	0	Swelling of explants, shoots formed in cluster from node
0	6	25	5^{d} (0.5)	2^{b} (0.2)	Shoot buds formed from node, few converted to rooted plantlets.
0	9	35	16^{b} (0.5)	0	Shoot buds light green, many converted to plantlets.
3	0	28	4^{d} (1.0)	5^{a} (0.3)	Plantlets with healthy roots, shoots etiolated and small leaves.
6	0	18	5^{d} (0.5)	4^{a} (0.2)	Shoot bud formed from node, few converted to plantlets with small leaves. Plantlet thin and elongated.
3	*3*	*21*	*18^{a} (1.5)*	*3^{b}(0.3)*	*Shoot bud formed in clusters, many converted to tiny plantlets.*
3	6	32	8^{c} (1.0)	1^{c}(0.2)	Shoot buds formed in cluster and all converted to plantlet.
6	3	18	4^{d} (0.5)	2^{b} (0.20	Shoots small, formed from node, unhealthy.
6	6	49	10^{c} (1.5)	2^{b} (0.20	Shoots/PLBs developed from node.
6	9	30	8^{c} (1.0)	0	Shoots formed from node in cluster. Few converted to plantlet with small leaves.
9	6	40	5^{d} (1.0)	0	Shoots formed at the node. Few shoots converted to healthy plantlets.

* Standard error, Values followed by the same *letters* are not significantly different from each other.

** On MS medium with 3% sucrose

Data represents the mean of 5 replicates

Data collected after 35 days of culture.

Only the significant treatment are computed

Figure 5: Culture of pseudobulb segments of *Malaxis acuminata* from *in vivo* and *in vitro* sources and their morphogenetic response. **a.** Shoot bud formation from the basal part of the pseudobulb segment cultured from *in vivo* source; **b.** Shoot buds formation from the basal part of the pseudobulb segment cultured from *in vitro* source; **c.** Multiple shoot buds and plantlets formed from the cultured pseudobulb segment; **d.** Multiple shoot buds and plantlets with roots formed from the pseudobulb segment cultured on activated charcoal enriched medium.

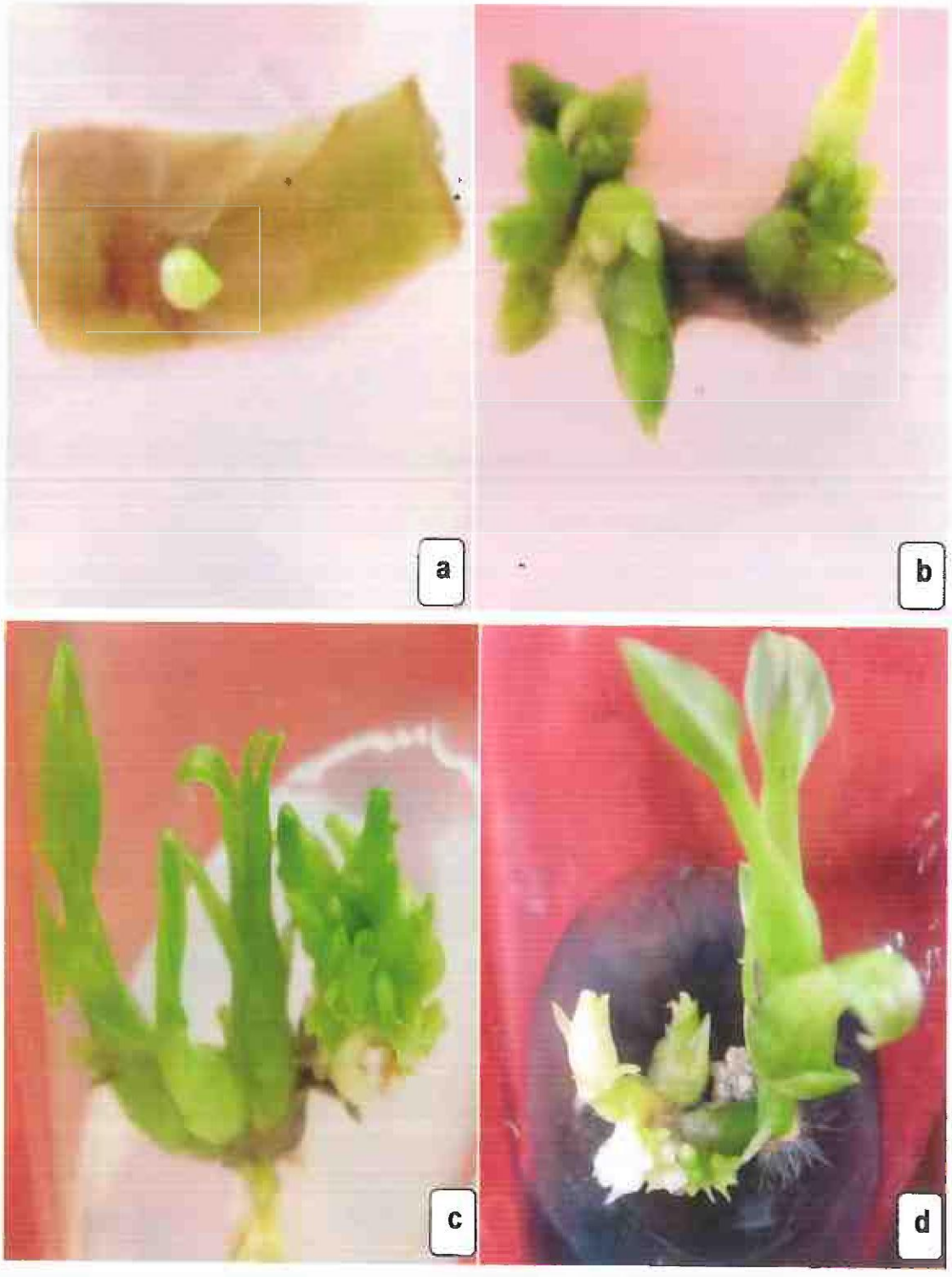

Figure- 5

Table 8: Effect of different organic carbon sources on pseudobulb culture of *Malaxis acuminata*

Organic Carbon Sources & Conc. (%)	No. of shoots formed	% response (±SE)*	Type of response**
0	0	0	No response
Dextrose			
1	3.3 ± 0.2^{d}	50.5 ± 1.5^{d}	Pseudobulb segments curled, small shoot buds formed from leaf scar.
2	7.2 ± 0.1^{b}	75.5 ± 2.0^{b}	Small shoot buds developed from leaf scar and base of the pseudobulb.
3	6.5 ± 0.1^{c}	60.0 ± 2.5^{c}	Shoots small, round, formed from leaf scar. One root formed. Shoot buds formed hairy structures
4	3.2 ± 0.2^{d}	50.4 ± 1.5^{d}	Shoot small, round formed from base, tip and leaf scar
Glucose			
1	2.2 ± 0.2^{e}	50.2 ± 3.0^{d}	Shoot buds small
2	4.0 ± 0.2^{d}	55.3 ± 2.0^{c}	As above
3	2.0 ± 0.1^{e}	50.5 ± 1.0^{d}	As above
4	5.2 ± 0.3^{c}	60.6 ± 1.2^{c}	Shoots formed from leaf scar. Shoot converted to plantlet. Hairy structures at base.
Sucrose			
1	4.3 ± 0.5^{d}	55.2 ± 1.5^{c}	Shoots developed from leaf scar and base
2	8.1 ± 0.5^{b}	75.2 ± 1.0^{b}	Healthy shoots
3	$\mathbf{11.0}\pm0.4^{a}$	$\mathbf{98.0}\pm1.0^{a}$	*Pseudobulb segment swollen, shoot buds/PLBs formed in cluster through out the segment*
4	7.4 ± 0.3^{b}	70.7 ± 1.0^{b}	Shoot very small, formed from leaf scar

* Standard error, Values followed by the same *letters* are not significantly different from each other.

**On MS medium containing NAA + BA (6+6 µM respectively in combination)

Data represents the mean of 5 replicates each

Table 9: Effects of PGRs on morphogenetic response of pseudobulb segments of *Malaxis acuminata* from *in vitro* source.

PGRs Conc. (µM) NAA	BA	% response (±SE*)	No. of shoot buds/ PLBs formed per segment*	Type of response**
0	0	-	-	-
3	0	30 ±0.5^{e}	3±0.4^{d}	PLBs formed from the cut ends
6	0	50±1.5^{d}	4±0.2^{d}	PLBs and shoot buds formed from cut ends
9	0	50±1.0^{d}	2±0.5^{d}	As above but degenerated
0	3	90±1.7^{b}	7±0.4^{b}	Shoot buds formed from all over the pseudobulb segment
0	6	90±2.0^{b}	6±.02^{c}	As above
0	9	75±1.5^{c}	6±0.4^{c}	As above but degenerated subsequently
3	3	55±0.6^{d}	8±0.5^{b}	Shoot buds formed at the tip and few converted into plantlets
6	3	45±1.5^{d}	5±0.2^{c}	As above but not healthy
9	3	43±2.0^{d}	4±0.2^{d}	As above
3	6	75±0.5^{c}	3±0.2^{d}	PLBs and shoot buds formed from the leaf scar and cut ends
6	*6*	*98*±1.0^{a}	*11*±0.4^{a}	*Pseudobulb segment swollen, shoot buds/PLBs formed in cluster through out the segment*
9	6	67±1.5^{c}	6±0.2^{c}	Cultures turned brown and degenerated
3	9	88±1.5^{b}	5±0.1^{c}	Shoot buds formed in cluster but degenerated
6	9	87±1.0^{b}	3±0.2^{d}	As above
9	9	67±2.0^{c}	3±0.4^{d}	As above

* Standard error, Values followed by the same *letters* are not significantly different from each other.

** On MS medium containing 3% sucrose, 100 mg L^{-1} CH and 100 mg L^{-1} citric acid.

Data represents the mean of five replicates.

Data scored after 70 days of culture;

many as 18 shoot buds/PLBs developed at a single node on MS medium supplemented with sucrose (3%) and NAA + BA (3+3 µM in combinations) (**Table 7, Fig. 4c**). About 90% explants responded positively with the sprouting of shoot buds/PLBs from the nodal regions under optimum conditions. Positive response was recorded with both single and combined treatments of NAA and BA.

Pseudobulbs

Besides immature embryos, foliar explants and nodal explants, cultures were also initiated from the pseudobulb segments both from *in vitro* as well as *in vivo* sources. In the preliminary study it was found that, of the two basal media tested, MS medium supported better morphogenetic response. Hence further studies were conducted only with MS medium. The explants from the two sources exhibited differential response. The pseudobulb segments from *in vivo* source were cultured on two different media containing different concentrations of NAA and BA. About 15% explants responded positively and an average of 2-3 shoot buds formed per segment on medium enriched with sucrose (3%) and NAA (6 µM) and BA (6 µM) in combination (data not presented) (**Fig. 5a**).

In comparison to pseudobulb from *in vivo* source, pseudobulb harvested from *in vitro* source exhibited a contrasting response. Within 7-8 wk of culture, the explants from the *in vitro* source developed multiple shoot buds/PLBs from the cut ends, leaf scar and tip of the explants (**Fig. 5b**). Of the three different organic carbon sources incorporated in different concentrations, sucrose (3%) supported optimum response (**Table 8**). As many as 11 shoot buds developed in ~98% of culture on MS medium containing sucrose (3%), NAA and BA (6 µM each in combination) (**Table 9, Fig. 5c**). Pseudobulb segments were also cultured on medium containing optimum PGRs and AC (0-0.4%) to study its effect on morphogenetic

Figure 6: Effect of activated charcoal on *in vitro* morphogenetic induction of pseudobulb segments of *Malaxis acuminata*.

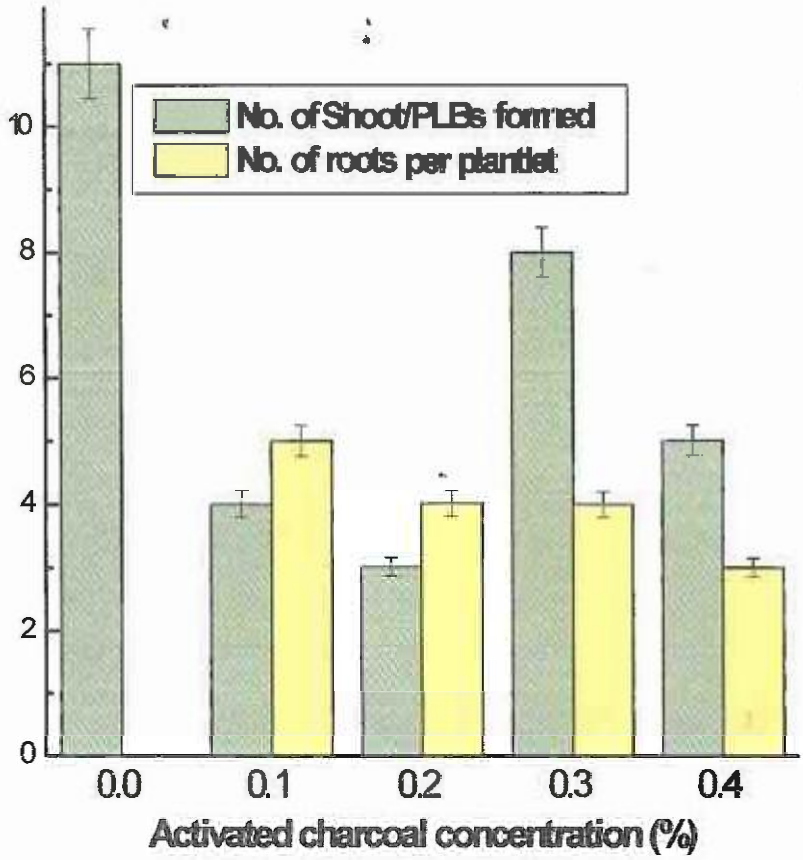

Figure 6: Effect of activated charcoal on *in vitro* morphogenetic induction of pseudobulb segments of *Malaxis acuminata*.

Figure 7: Effect of media and different alternate substrata on *in vitro* morphogenetic induction of pseudobulb segments of *Malaxis acuminata*.

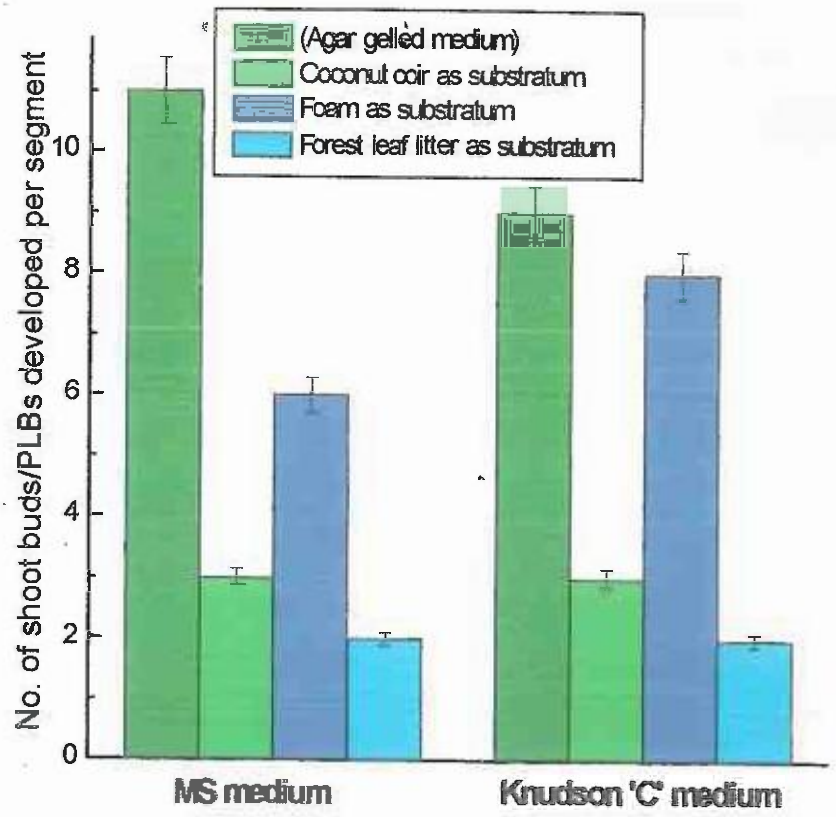

Figure 7: Effect of media and different alternate substrata on *in vitro* morphogenetic induction of pseudobulb segments of *Malaxis acuminata*.

response. Incorporation of AC in the medium did not support healthy morphogenetic response. On AC control medium there were as many as 11 shoot bud/PLBs formation while on AC enriched medium under optimum condition (0.3% AC) there were only 8 shoot bud formation (**Fig. 6**). Though incorporation of AC in the medium did not support optimum shoot buds/PLBs formation but it supported invocation of roots in the shoot buds (**Fig. 5d**). As many as 5 roots developed per plantlets while there was no root formation in AC control medium.

Besides agar gelled medium, the pseudobulb segments from *in vitro* source were also maintained on three other substrata viz., foam, coconut coir and forest leaf litter in two different basal media i.e., MS and Knudson 'C' media. It was observed that in both the media, agar gelled media supported better morphogenetic response followed by foam as substrata (**Fig. 7**). While, coconut coir and forest litter as substrata did not support healthy response.

The PLBs and shoot buds formed from the cultured immature embryos/seeds, foliar explants, nodal segments and pseudobulbs were maintained for another two passages for further differentiation and proliferation on optimum growth conditions. The advanced stage PLBs, shoot buds developed from the germinated seeds, foliar explants and cultured nodal explants and pseudobulbs were transferred on regeneration medium for regeneration of plantlets and culture proliferation.

Plantlet regeneration and culture proliferation

The advanced stage PLBs/shoot buds developed from different explants were maintained further for two passages on the optimum initiation conditions for further differentiation.

Table 10: Effect of basal media and PGRs on plant regeneration and culture proliferation of *M. acuminata

PGRs Conc. (µM)		Plant height (cm)#		No. of leaves#		No. of shoots#		No. of roots#	
NAA	BA	*M*	*Mi*	*M*	*Mi*	*M*	*Mi*	*M*	*Mi*
0	0	-	-	-	-	-	-	-	-
0	3	2.1±0.3^{b}	1.3±0.2^{b}	3.2±0.3^{c}	3.3±0.2^{a}	3.2±0.4^{d}	2.0±0.3^{e}	1.0±0.1^{d}	1.0±0.1^{c}
0	6	1.5±0.2^{d}	1.5±0.1^{a}	2.0±0.2^{d}	2.0±0.1^{c}	4.3±0.3^{d}	8.1±1.0^{b}	1.0±0.2^{d}	1.0±0.2^{c}
0	9	1.6±0.3^{c}	1.4±0.1^{b}	3.1±0.3^{c}	2.0±0.1^{c}	8.5±0.5^{c}	6.3±0.5^{c}	1.0±0.1^{d}	2.0±0.2^{b}
3	0	2.4±0.2^{b}	1.2±0.2^{b}	3.2±0.1^{c}	2.3±0.3^{c}	2.0±0.3^{e}	10.5±1.5^{a}	1.0±0.1^{d}	2.0±0.1^{b}
6	0	3.1±0.3^{a}	1.1±0.2^{c}	3.2±0.2^{c}	2.1±0.2^{c}	3.1±0.4^{d}	8.3±1.0^{b}	4.0±0.1^{b}	3.0±0.3^{a}
9	0	1.1±0.2^{d}	1.0±0.1^{c}	2.0±0.1^{d}	2.0±0.2^{c}	2.0±0.2^{e}	6.1±1.1^{c}	1.0±0.2^{d}	1.0±0.1^{c}
3	***3***	***2.4***±0.1^{b}	1.5±0.3^{a}	***4.5***±0.2^{a}	3.1±0.3^{a}	***18.0***±1.0^{a}	3.0±0.5^{d}	***4.0***±0.2^{b}	2.0±0.2^{b}
3	6	1.9±0.4^{c}	1.6±0.3^{a}	3.0±0.2^{c}	3.0±0.3^{b}	6.1±1.0^{c}	7.1±0.3^{b}	4.0±0.3^{b}	2.0±0.1^{b}
3	9	1.8±0.2^{c}	0.8±0.2^{c}	2.1±0.1^{d}	3.2±0.4^{a}	3.1±0.5^{d}	5.5±0.5^{c}	1.0±0.1^{d}	1.0±0.1^{c}
6	3	2.9±0.3^{a}	1.5±0.1^{a}	4.2±0.2^{b}	2.1±0.3^{c}	9.2±1.5^{b}	5.5±0.0^{c}	6.0±0.4^{a}	2.0±0.1^{b}
6	6	1.6±0.1^{c}	1.2±0.1^{b}	3.1±0.3^{c}	2.1±0.2^{c}	10.1±1.5^{b}	6.3±0.5^{c}	2.0±0.2^{c}	1.0±0.1^{c}
6	9	1.9±0.1^{c}	1.2±0.1^{b}	3.1±0.3^{c}	2.1±0.3^{c}	8.5±1.0^{c}	5.3±0.3^{c}	2.0±0.2^{c}	1.0±0.1^{c}
9	3	1.7±0.2^{c}	1.4±0.2^{b}	3.1±0.2^{c}	2.1±0.1^{c}	10.3±1.0^{b}	6.5±0.4^{c}	1.0±0.1^{d}	1.0±0.1^{c}
9	6	2.1±0.3^{b}	1.5±0.3^{a}	3.1±0.2^{c}	2.0±0.2^{c}	3.0±0.5^{d}	2.0±0.2^{e}	0.0	0.0
9	9	1.8±0.3^{c}	1.3±0.2^{b}	2.1±0.2^{d}	2.0±0.1^{c}	8.3±0.5^{c}	4.1±0.3^{d}	0.0	0.0

* Media containing sucrose (3%) and AC (0.3%).

M: On MS medium, *Mi*: On Mitra *et al* medium.

Data represents the mean of five replicates and data collected after 70 day of culture on the medium.

Values followed by the same *letters* are not significantly different from each other.

Table 11: Regeneration of *M. acuminata* on different strength of MS medium

Different Strength of MS medium	Height of plantlet (cm)	No. of leaves	No. of shoots	No. of roots	Type of response*
1/4	1.4	4	1	2	Leaves small, dark green Pigmentation developed at the base
1/2	1.8	4	5	4	Leaves small, dark green, opened. Pigmentation developed at the base. Hairy roots
3/4	2.1	5	9	3	Leaves small, dark green, opened. Plantlets healthy.
Full	2.4	5	18	4	Healthy plantlets with many shoot buds, violet pigmentations formed, pseudo bulb swollen

* On MS medium containing sucrose (3%) and NAA+BA (3 μMeach in combination)

Data harvested after 40 days of culture

Data represents the mean of 5 replicates

Table 12: Effect of different organic carbon sources on plant regeneration and culture proliferation of *M. acuminata.**

Source of organic carbon & Conc.(%)	Avg. Plantlet height (cm)**	Avg. No. of leaves per plant**	Avg. No. of shoots formed per subculture**	Avg. No. of roots per plant**
0	0	0	0	0
Dextrose				
1	1.3±0.20^{d}	3.5±0.10^{c}	06.33±0.27^{c}	1.5±0.10^{f}
2	2.0±0.15^{b}	4.3±0.15^{b}	10.50±0.15^{b}	2.6±0.20^{e}
3	1.7±0.20^{c}	3.3±0.13^{c}	03.33±0.10^{d}	2.6±0.10^{e}
4	1.6±0.10^{c}	2.5±0.15^{d}	02.50±0.12^{d}	1.5±0.10^{f}
Glucose				
1	1.4±0.05^{d}	3.5±0.10^{c}	03.00±0.10^{d}	4.3±0.20^{c}
2	1.4±0.10^{d}	2.5±0.20^{d}	06.50±0.20^{c}	4.6±0.15^{b}
3	1.6±0.15^{c}	2.5±0.15^{d}	05.66±0.24^{c}	5.5±0.10^{a}
4	1.4±0.10^{d}	2.3±0.10^{d}	03.33±0.25^{d}	3.3±0.20^{d}
Sucrose				
1	1.5 ±0.15^{d}	3.5±0.15^{c}	07.50±0.50^{c}	3.0±0.15^{d}
2	1.5±0.10^{d}	3.3±0.20^{c}	10.00±0.50^{b}	4.3±0.25^{c}
3	2.4±0.10^{a}	4.5±0.20^{a}	18.00±1.00^{a}	4.0±0.20^{b}
4	2.0±0.10^{b}	4.0±0.20^{b}	06.33±1.00^{c}	2.5±0.10^{e}

* On MS medium containing NAA and BA (3 + 3 µMin combination) and AC (0.3%).

Data represents the mean of five replicates and data colected after 70 day of culture on the medium.

** Standard error. Values followed by the same *letters* are not significantly different from each other.

Effect of basal media The differentiated PLBs/shoot buds and young plantlets were maintained on two different media viz., Mitra *et al* and MS media with different growth adjuncts. In the preliminary study it was observed that MS medium supported better regeneration and multiplication of culture (**Table 10**) compared to the Mitra *et al* media. The cultures maintained on MS medium differentiated faster and supported higher number of secondary shoots/PLBs formation. Subsequent experiments were conducted to study the effects of different strengths of MS medium on plant regeneration and plant growth. Amongst the different strengths of MS medium studied in the present investigation, it was found that at lower strengths ($1/4^{th}$, 1/2 strengths) plant growth was stunted accompanied by fewer new shoot bud formation (**Table 11**). Optimum plant height (~2.4 cm) as well as maximal shoot buds formation (18 shoot buds per plant per subculture) were achieved on full MS medium (**Table 10, 11, Fig. 2c**). At lower strengths of MS medium, plants exhibited stunted growth along with fewer leaves and roots.

Effect of different organic carbon sources: The basal medium was fortified with different quality and quantity of organic carbon sources like dextrose, glucose and sucrose (0-4%). Of the three different organic carbon sources incorporated in the regeneration medium, in general cultures maintained on sucrose enriched medium outperformed the cultures maintained on medium with other organic carbon sources under otherwise identical conditions. The cultures maintained on glucose enriched medium did not support regeneration and cultures degenerated subsequently while, dextrose nourished medium supported poorer regeneration with fewer secondary shoot buds formation. Under the conditions provided, the optimum culture differentiation and plant regeneration was achieved when MS medium was conjunct with sucrose (3%) (**Table 12**). Under this condition as many as 18 shoot buds formed per

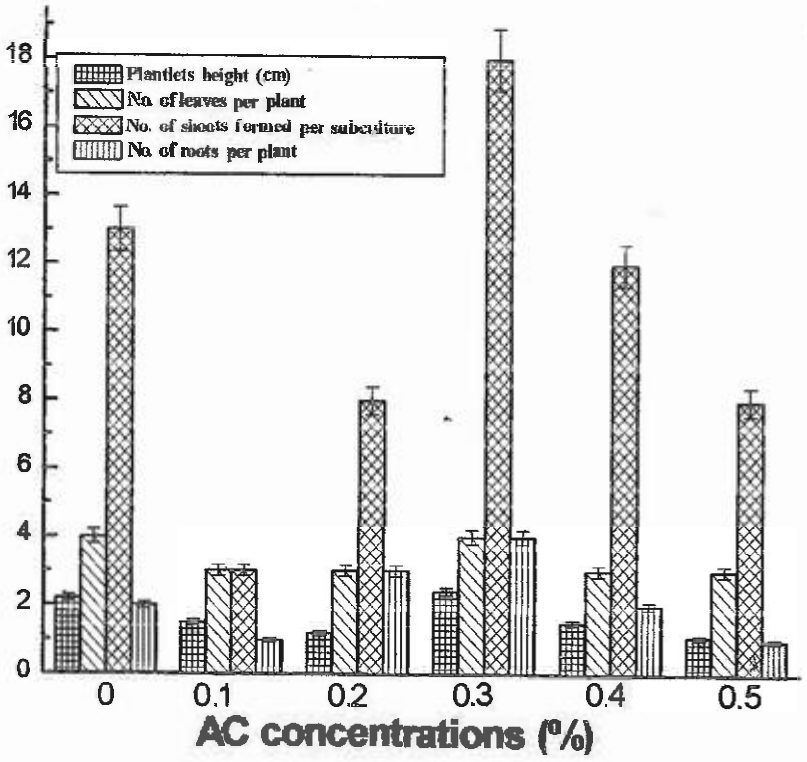

Figure 8: Effect of activated charcoal on plant regeneration, culture proliferation of *M. acuminata*.

plant per subculture where the average height of the plantlets, number of leaves and number of roots were 2.4 cm, 4.5 and 4.0 respectively (**Fig. 2c**).

Effect of PGRs: The advanced stage PLBs from cultured seeds started converting into young rooted plantlets and repetitive PLBs within 3-4 wk on regeneration media. The different quality and quantity of PGRs incorporated in the regeneration medium exhibited differential response on regeneration and mass multiplication. It was observed that the single treatments of different PGRs at lower concentrations resulted stunted growth of the regenerates while at higher concentrations, the regenerates showed etiolated growth. None of the single concentration treatment could support the formation of multiple shoots and only the combined treatments of different PGRs produced multiple shoots/propagules. Amongst the different quality and quantity of PGRs used, NAA and BA (3+3 µM in combination) supported optimum regeneration and mass multiplication of plantlets which produced multiple shoots/buds. As many as 18 shoots/shoot buds developed per explants per subculture (**Table 10**).

Effect of activated charcoal: Apart from all the optimum conjuncts, AC was also incorporated at different concentrations (0-0.5%) in the regeneration medium. Incorporation of AC in the multiplication medium facilitated the culture proliferation, induction of newer shoot buds and enlargement of pseudobulbs. While under AC control there was only about 13 shoot buds/PLBs formation (**Fig. 8**) in comparison to ~18 shoot buds formation on AC (0.3%) enriched medium. It was observed that, when cultures were maintained on AC enriched medium, it accelerated plant regeneration, culture proliferation and pseudobulb enlargement and root formation. The optimum response was achieved on MS medium enriched with

Figure 9: Plantlets regeneration and multiple shoot buds formation of *Malaxis acuminata* on regeneration medium containing different substrata. **a**. Multiple shoot buds/PLBs formed on regeneration medium containing foam as substrata; **b**. Multiple shoot buds/PLBs formed on agar gelled regeneration medium; c. Multiple shoot buds/PLBs formed on regeneration medium containing coconut coir as substrata; **d**. Multiple shoot buds/PLBs formed on regeneration medium containing forest leaf litter as substrata.

Figure- 9

Table 13: Effects of PGRs and different substrata on plantlet regeneration and mass multiplication of *Malaxis acuminata*#.

PGRs Conc. (µM)		Plantlet height (cm)*				No. of leaves per plantlet*@				No. of shoots formed per subculture*@			
NAA	BA	*A*	*F*	*C*	*L*	*A*	*F*	*C*	*L*	*A*	*F*	*C*	*L*
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	3	2.1±0.3^{b}	1.7±0.2^{c}	1.3±0.1	1.6±0.1	3.2±0.3^{c}	3.3±0.2^{c}	2.2±0.1^{d}	3.0±0.2b	3.2±0.4^{d}	11.0±1.5^{b}	2.2±0.1^{c}	1.0±0.1^{c}
0	6	1.5±0.2^{d}	1.4±0.4^{d}	1.3±0.2	1.5±0.2	2.0±0.2^{d}	3.0±0.3^{c}	3.3±0.3^{c}	4.2±0.2^{a}	4.3±0.3^{d}	8.5±0.5^{cd}	4.0±0.2^{b}	1.0±0.2^{c}
0	9	1.6±0.3^{c}	1.2±0.3^{d}	1.3±0.1	1.4±0.2	3.1±0.3^{c}	2.5±0.3^{c}	3.0±0.2^{c}	3.2±0.1^{b}	8.5±0.5^{c}	6.5±0.5^{e}	5.0±0.2^{b}	1.0±0.1^{c}
3	0	2.4±0.2^{b}	1.5±0.2^{d}	1.3±0.2	1.3±0.2	3.2±0.1^{c}	4.2±0.4^{b}	3.0±0.2^{c}	3.3±0.2^{b}	2.0±0.3^{e}	8.0±0.7^{d}	2.0±0.1^{c}	1.0±0.2^{c}
6	0	3.1±0.3^{a}	2.2±0.3^{b}	1.2±0.3	1.7±0.2	3.2±0.2^{c}	3.5±0.2^{c}	2.5±0.3^{d}	3.0±0.1^{b}	3.1±0.4^{d}	10.0±1.5^{c}	1.0±0.1^{d}	1.0±0.1^{c}
9	0	1.1±0.2^{d}	1.1±0.3^{d}	1.0±0.1	1.2±0.1	2.0±0.1^{d}	2.4±0.1^{d}	2.2±0.1^{d}	2.5±0.2^{c}	2.0±0.2^{e}	6.5±1.0^{e}	1.0±0.2^{d}	1.0±0.2^{c}
3	***3***	***2.4*±0.1**^{b}	***2.2*±0.2**^{b}	***2.2*±0.2**	1.8±0.2	***4.5±0.2***^{a}	5.0±0.3^{b}	5.0±0.2^{b}	3.0±0.2^{b}	***18.0±1.0***^{a}	***14.0*±0.5**^{a}	3.5±0.2^{c}	3.0±0.2^{c}
3	6	1.9±0.4^{c}	1.5±0.2^{d}	1.2±0.3	1.2±0.1	3.0±0.3^{c}	3.0±0.4^{c}	**6.0±0.3**^{a}	3.0±0.1^{b}	6.1±1.1^{c}	12.0±0.7^{b}	**8.0**±0.4^{a}	2.0±0.2^{b}
3	9	1.8±0.2^{c}	1.4±0.3^{d}	1.2±0.2	1.2±0.1	2.1±0.1^{d}	3.0±0.2^{c}	3.0±0.2^{c}	2.5±0.1^{c}	3.1±0.5^{d}	5.0±0.5^{e}	2.0±0.2^{c}	-
6	3	2.9±0.3^{a}	1.6±0.2^{c}	1.0±0.2	1.1±0.1	4.2±0.2^{b}	4.5±0.2^{b}	3.0±0.1^{c}	2.5±0.1^{c}	9.2±1.5^{b}	12.0±1.0^{b}	5.0±0.3^{b}	-
6	6	1.6±0.1^{c}	3.0±0.4^{a}	0.9±0.1	1.3±0.1	3.1±0.3^{c}	**6.2**±0.4^{a}	2.0±0.2^{d}	3.0±0.1^{b}	10.1±1.5^{b}	11.5±1.5^{b}	1.0±0.2^{d}	-
6	9	1.9±0.1^{c}	2.5±0.2^{b}	1.1±0.3	1.0±0.1	3.1±0.3^{c}	3.0±0.2^{c}	2.0±0.1^{d}	2.0±0.1^{c}	8.5±1.0^{c}	9.0±0.5^{c}	-	-
9	3	1.7±0.2^{c}	2.2±0.1^{b}	1.2±0.2	-	3.1±0.2^{c}	3.2±0.3^{c}	3.1±0.2^{c}	3.0±0.2^{b}	10.3±1.0^{b}	9.0±1.0^{c}	-	-
9	6	2.1±0.3^{b}	2.0±0.3^{bc}	1.1±0.2	-	3.1±0.2^{c}	3.0±0.2^{c}	2.5±0.2^{d}	3.0±0.1^{b}	3.0±0.5^{d}	5.5±0.6^{e}	-	-
9	9	1.8±0.3^{c}	1.2±0.3^{d}	-	-	2.1±0.1^{d}	2.0±0.1^{d}	2.0±0.1^{d}	2.0±0.1^{e}	8.3±0.5^{c}	-	-	-

In MS medium containing 3% sucrose; Data represent the mean of five replicates; * **A**: Agar gelling medium, **F**: Foam as substratum, **C**: Coconut coir as substratum, **L**: Forest litter as substratum; @ Standard error. Values followed by the same *letters* are not significantly different from each other.

Figure 10: Hardening of regenerated plants of *Malaxis acuminata* and transplanting to community potting mix. **a**. One of the well rooted plant; **b**. A rooted plant on hardening condition; **c**. Hardened plants transferred to community potting mix.

Figure - 10

sucrose (3%), NAA and BA (3 µM each in combination) and AC (0.3%) where average 4.5 leaves, 4 roots per plants and 18 shoot buds were formed per subculture.

Effects of different alternative substrata on regeneration and culture proliferation In the present study, foam was successfully used as alternative to agar for regeneration and culture differentiation. Amongst the different substrata incorporated in the regeneration medium, better regeneration and multiple shoot buds formation were registered on medium containing foam disk as substratum and agar gelled medium (**Table 13, Fig. 9a, b**). Of the different substrata, coconut coir and forest leaf litter were found to be inferior compared to agar and foam disk (**Fig. 9c, d**). As many as 18 shoot buds developed on agar gelled medium against 14 shoot buds on foam disk. On media with coconut coir and forest leaf litter as substrata, the number of shoot buds produced was much lower than on agar gelled and foam containing media (**Table 13**). Besides this, cultures maintained on these two conditions required prolonged period for differentiation.

The regenerated plantlets were maintained on optimum regeneration condition for 2-3 passages for culture proliferation and plantlet growth. About 3 cm long plantlets with distinct pseudobulb, 4-5 leaves and 4-5 roots from regeneration medium were taken out from the regeneration medium and subjected to *in vitro* hardening (**Fig. 10a**).

Hardening and field trial of the regenerates

The well developed plants with characteristic pigmentation of *Malaxis acuminata* were transferred to ½ strength MS medium containing sucrose (2%) but freed from any PGRs and maintained for ~6-7 wk under normal laboratory condition (**Fig. 10b**). The hardened plants were transferred to CPM (**Fig. 10c**). To transplant the hardened plantlets, the CPM was

prepared by mixing different substrates like charcoal pieces, chopped forest litters, coconut husk, sand and black soil (at 1:1 ratio) with a moss topping. The hardened plants were transferred to CPM which was then covered with holed transparent poly bags. The potted plants were maintained in poly house (ca 70% filtered light) and fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 wk. The potted plants were left in the normal full day light conditions which were kept for about 7-8 wk before transferring to the wild. During this process plantlets turned deep green and developed pigments. About ~75% of the transplants survived to form fully developed plants after two months of potting.

2.3. Discussion

Initiation of culture

Immature seeds/embryos

A single orchid capsule contains millions of seeds which are microscopic and lack any metabolic machinery. As orchid seeds possess little or no endosperm, their natural germination is limited and the development of seeds in orchid is very poor even at maturity. They need a symbiotic association with specific mycorrhizal fungus which provides an essential physico-chemical stimulus for initiating germination (Harley, 1959, Pongener and Deb, 2011a). This fungal requirement can, however, be compensated by the *in vitro* incorporation/supplements of sugars/different organic carbon sources and adjuvants. Knudson (1922) demonstrated the possibility of by passing the fungal requirement of orchid seeds during *in vitro* germination and since then asymbiotic/non-symbiotic seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids (Arditti *et al*., 1982). *In vitro* germination of orchid seeds is an important part of the

propagation and conservation programme, as the '*dust seeds*' are tiny and contain limited food reserves. The fertilized ovules/immature embryos are used successfully for micropropagation and rapid mass multiplication of several commercially viable and or threatened orchids (Devi *et. al.*, 1998; Pathak *et. al.*, 2001; Sharma and Tandon, 1990; Temjensangba and Deb, 2005a, c). The orchids are also propagated by vegetative means like keikis and or shoot buds.

The successful non-symbiotic seed germination and or immature embryo culture of orchids are greatly influenced by several factors like seed pod age, different nutrient media, different media supplements, plant growth regulators 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). However, the media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the cultured immature embryos/seeds without the intermediary fungus (Mc Kendrick, 2000). But none of these basal nutrient media with different adjuvant fulfill the requirements of the entire orchidaceous group.

Effects of basal media on immature seeds germination: In the present investigation, two different basal media viz., Mitra *et al* and MS were used for culture initiation from immature seeds/embryos. Of the two media tested, optimum germination was achieved on MS basal medium. Under optimum condition a germination rate of ~85% was achieved on MS medium followed by Mitra *et al* (65%) media. The orchids of different species exhibit a preferential requirement to specific nutrient media for seed germination but as such no standard media can be prescribed for all the orchid 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), *Habenaria macroceratitis* on LC and KC media (Stewart and Kane, 2006), *Rhynchostylis gigantea* on MS medium (Li and Xu, 2009), *Taenia latifolia* on MS medium (Deb and Sungkumlong, 2008), *Vanda coerulea* in Ichihashi & Yamashita (Rao *et al*., 1998) and VW media (Devi *et al*., 1998), *Aerides rosea* in Knudson 'C', VW and MS media (Sinha *et al*., 1998). In the present study, with *Malaxis acuminata* MS medium was found to be superior for *in vitro* seed germination. A similar response was also reported with other orchid species like *Cymbidiums* (Nagaraju and Upadhyaya, 2001), *Geodorum densiflorum* (Sheelavanthmath *et. al*., 2000) and *Renanthera imschootiana* (Laishram and Devi, 1999).

Effects of green pod age on in vitro culture of seeds A key factor for successful non-symbiotic seed germination in the present study was the physiological age of the green pod/capsule. Different species of orchids 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 orchid species, which supports optimum *in vitro* germination. The earliest stage at which the embryos can be cultured successfully varies within the orchid 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 stage to harvest the green capsule/pods to achieve an optimal germination. The culture of immature green pod/embryos ensures sterility but may require prolong period for germination as the seeds are immature. While, the culture of comparatively mature capsules/embryos before dehiscing may support better germination but the chances of contamination increases due to establishment of mycorrhizal association (Mc Kendrick, 2000). In the present investigation, the green pod age of 7-8 WAP supported optimum germination (85%). The green pod age <6 WAP either did not germinate or exhibited delayed germination while, seeds age >9 WAP, germination frequency was comparatively lower. In *Dacylorrhiza hatagirea*, seeds of 16 WAP (Vij *et al*., 1995), in *Malaxis khasiana*, seeds of 8 WAP (Deb and Temjensangba, 2006a), in *Rhynchostylis gigantea*, 4 months old seeds (Li and Xu, 2009) exhibited better germination but 12 WAP was ideal planting materials in *Cymbidium macrorhizon* (Vij and Pathak, 1988). The ability of immature embryos to germinate 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).

Effect of organic carbon sources: Three different organic carbon sources at differential concentrations (viz. dextrose, glucose and sucrose at 0-4%) were incorporated in the medium for *in vitro* culture of immature embryos. It was observed that incorporation of at least one of

the organic carbon sources was prerequisite for successful *in vitro* culture of immature embryos/seeds. In absence of organic carbon source, only nodular swellings of embryos were observed. The swelled seeds failed to convert into PLBs and degenerated subsequently. Presence of organic carbon sources such as dextrose, glucose and sucrose in the initiation media showed a marked effect on the germination of seeds. The different levels of the organic carbon sources showed differential effect on seed germination. 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). Sharma and Tandon (1990) reported that 2-3% sucrose, D-Fructose and D-Glucose were the suitable organic carbon sources for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata* while, 3% sucrose was found to be suitable for immature seed germination of *Geodorum densiflorum* (Bhadra and Hossain, 2003). In the present study, amongst the different organic carbon sources tested, optimum seed germination of *M. acuminata* was obtained on basal media containing 3% sucrose. Both dextrose and glucose enriched medium delayed germination compared to sucrose enriched medium. Amongst the three sources of organic carbon tested, glucose proved to be least preferred but in *Cymbidium aloifolium* and *C. iridioides* dextrose was least preferred (Pongener and Deb, 2009, 2011a).

Effect of light intensity. In the present study, different light intensity exhibited differential effect on nodulation of seeds, per cent germination and time for PLBs formation. Though cultures maintained under dark condition swelling of seeds started within 38 days of culture against 50 and 55 days under diffused light and full light conditions respectively (**Table 4**) but the seeds failed to form PLBs in the same speed. Cultures maintained in the full light

condition supported early PLBs formation (135 days) against 170 and 148 days under dark and diffused light conditions. In *C. aloifolium* and *C. iridioides*, cultures maintained in dark exhibited early nodulation but, optimum germination (90% in *C. aloifolium* and 95% in *C. iridioides*) was registered under full light (40 µmol m^{-2} s^{-1}) compared to cultures maintained in the dark and diffused light (20 µmol m^{-2} s^{-1}) conditions (Pongener and Deb, 2009, 2011a). While, in *Arachnis labrosa* (Temjensangba and Deb (2005a) and in *C. racemiferum* (Deb and Temjensangba, 2007b, Temjensangba and Deb, 2006), diffused light condition of 20 µmol m^{-2} s^{-1} was found suitable for initiation of asymbiotic germination followed by full light 40 µmol m^{-2} s^{-1}) conditions at 12/12 h photoperiod. But in *Habenaria macroceratitis* (Stewart and Kane, 2006) 8/16 hr (L/D) photoperiod was preferred over 0/24 and 12/12 hr photoperiod. On the other hand Deb and Temjensangba (2006a) observed that diffused light condition supported higher rate of germination in *Malaxis khasiana*. Islam *et al.* (2003) reported that the *Phalaenopsis* callus growth and development of PLBs were better in illumination than in dark. The effect of light for embryogenic culture initiation in conifers is well documented (Deb and Tandon, 2004; Gupta and Grob, 1995; Von Arnold, 1987). In *Pinus kesiya*, light was reported inhibitory for initiation of embryogenic cultures and produced more non-embryogenic cultures. Dark was optimum while diffused light formed moderate embryogenic cultures (Deb and Tandon, 2004).

Effect of PGRs. In the present investigation, even in the absence of PGRs, nodular swellings of the immature embryos were possible but failed to differentiate further. The PGRs in the germinating medium showed a marked effect on the growth, differentiation and development of PLBs. Although BA and NAA had stimulated the germination of immature embryos of *M. acuminata*, NAA was more effective then BA (**Table 1**). The germination was accelerated by

addition of NAA to the medium. Seed germination of *M. acuminata* on NAA enriched medium was evident after 8 wk of culture and formed PLBs after 135 days of culture. While, seeds maintained on BA rich medium either singly or in combination with NAA delayed germination and germination frequencies were also significantly lower. 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).

Initiation of culture from leaf

In the current investigation cultures were also initiated from the foliar explants from *in vitro* source. Wimber (1965) successfully developed PLBs from the leaves of *Cymbidiums*, which opened up an effective alternative to apical shoot meristem culture. Since then the regenerative competence of foliar explants were positively tested for more than 60 orchid species (Temjensangba and Deb, 2005b). However, the success is restricted mostly with epiphytic orchids and only few species from terrestrial orchids suggesting thereby that the ground orchids are less amenable to *in vitro* regeneration (Deb and Sungkumlong, 2010). In the present study, initiation of morphogenetic response was restricted to the basal parts of the foliar explants. The morphogenetic potential of leaf base has been reported in *Coelogyne*, *Dendrobium*, *Oncidium* and *Phalaenopsis* (Abdul Karim and Hairani, 1990), *Acampe praemorsa* (Nayak *et al.*, 1997), *A. labrosa* (Deb and Temjensangba, 2007a), *C. racemiferum* (Temjensangba and Deb, 2005b), *C. suaveolens* and *Taenia latifolia* (Deb and Sungkumlong,

2010), *V. coerulea* (Vij and Aggarwal, 2003). Mathews and Rao (1985) considered the leaf base to be the decisive factor for culture initiation from foliar explants. Sinha and Hegde (1999) reported the development of meristematic activity along the entire leaf in Renades Arunoday Hybrid. In the present study the orientation of explants was observed to be another crucial factor for morphogenetic response. Explants cultured horizontally exhibited better response compared to upside up and upside down orientation (Table 5). Earlier, Nayak *et al.* (1997) in *Acampe praemorsa* reported the influence of explants orientation of shoot development.

The incorporation of PGRs to the basal medium was obligatory for the initiation of culture. The explants failed to respond, when cultured on PGRs free medium. The role of growth hormones in stimulating meristematic activity and promoting proliferation in leaf explants is well documented in orchids (Abdul Karim and Hairani, 1990; Vij and Pathak, 1990; Yam and Weatherhead, 1991; Arditti and Ernst, 1993; Vij *et al.*, 1994; Nayak *et al.*, 1997; Temjensangba and Deb, 2005b; Deb and Temjensangba, 2007a; Li and Xu, 2009; Deb and Sungkumlong, 2010). Murashige (1974) opined that *in vitro* plant regeneration occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. In *M. acuminata* meristematic loci invoked after about 28 days of culture initiation where as many as 26 shoot buds/PLBs formed per explants in about 65% of cultures (**Table 6**). Amongst the different PGRs tested, optimum response was registered on MS medium containing sucrose (3%), NAA and BA (3 and 6 µM respectively in combination).

Initiation of culture from nodal explants

In the present study, cultures were also initiated with nodal segments of *M. acuminata* from *in vitro* source. Morphogenetic response and sprouting of shoot buds/PLBs from the

nodal region of the segments were initiated within 21 days of culture. A combined treatment of NAA and BA was more effective over single treatment of either of the PGRs for culture differentiation (**Table 7**). About 18 shoot buds/PLBs developed from each node on MS medium supplemented with sucrose (3%) and BA + NAA (3 µM each) in combination. Present study is in agreement with the reports in *Phalaenopsis* where a combined treatment of BA (2 mg L^{-1}) and NAA (0.5 mg L^{-1}) was found to be optimum for breaking axillary buds and formation of multiple shoot buds (Kosir *et al*, 2004) and observed that incorporation of NAA was promotory. Other workers like Tisserat and Jones (1999), Roy and Banerjee (2003) also observed that an appropriate combination of NAA and BA stimulated multiple shoot buds formation. But, Arditti and Ernst (1993), Pongener and Deb (2011b) reported that BA promote morphogenetic response but addition of NAA reduced induction and regeneration.

Initiation of culture from pseudobulbs segments

Besides other explants, cultures were also initiated from the pseudobulb segments from both *in vitro* and *in vivo* sources. The explants from the two sources exhibited differential response. The formations of meristematic loci were registered through out the cultured segments of pseudobulbs from *in vitro* source. But, invocation meristematic loci were restricted from the leaf scar in limited numbers of explants (~15%) in the segments collected from the *in vivo* sourced. The pseudobulb segments from *in vitro* source produced as many as 11 shoot buds on medium containing NAA and BA (6+6 µM respectively in combination). While the explants from *in vivo* source, resulted 2-3 shoot buds after 10 wk of culture when enriched with NAA and BA (6+6 µM respectively in combination) but degenerated subsequently. Under optimum condition, ~98% explants responded positively. The healthy shoot bud induction was registered on medium supplemented with both NAA and

BA. The present study is in line with the reports of (Seeni and Latha, 1992; Vij and Aggarwal, 2003; Deb and Temjensangba, 2006a, b; Sungkumlong and Deb, 2009). Addition of AC in the medium proved to be inhibitory for culture initiation. Earlier AC was used positively for culture initiation in *Coelogne viscosa* (Vij *et al*, 1997b), *Malaxis khasiana* (Deb and Temjensangba, 2006a), *Taenia latifolia* (Sungkumlong and Deb, 2009).

Effect of different alternative substrata. Apart from agar, other materials like coconut coir, foam disk and leaf litter could be successfully used with differential success for culture initiation from pseudobulb segments. In *M. acuminata*, medium gelled with agar supported highest number of shoot buds formation followed by on foam as substratum. As many as 11 shoot buds formed on agar gelled medium against ~6 shoot buds on foam as substratum. But the response on coconut coir and forest litter was far below compare to other two substrata.

Regeneration of plantlets and culture proliferation

The PLBs and shoot buds formed from the cultured immature embryos/seeds, foliar explants, nodal segments and pseudobulb segments were maintained for another two passages for further differentiation and proliferation on optimum initiation conditions. The advanced stage PLBs, shoot buds, tiny plantlets developed on initiation medium were transferred on regeneration medium for plantlets formation and culture proliferation.

Effect of basal media: In the preliminary study, the PLBs/shoot buds and tiny plantlets were maintained on two different basal media viz., Mitra *et al* and MS in conjunction with different adjuvant for regeneration of plantlets and mass multiplication. The PLBs/shoot buds differentiated into plantlets after 2-5 wk of transfer. Amongst the two media tested, MS medium supported highest numbers of plantlets formation and better culture proliferation

whereas; Mitra *et al* medium supported poorer culture growth, culture differentiation and proliferation.

Further experiments were conducted to study the effect of different strengths of MS medium on regeneration of plantlets and culture proliferation. It was found that full strength MS medium supported the formation of maximum well rooted plantlets, shoot buds and secondary PLBs. At lower strength of MS medium ($1/4^{th}$ and 1/2 strengths) most of the plantlets were either stunted in growth with fewer new shoot buds/PLBs formation or cultures degenerated. At $3/4^{th}$ strength of MS medium the plantlet height was comparatively better than $1/4^{th}$ 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 seedlings. 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 in orchids and such behavior can be judiciously exploited to achieve desirable response in many orchid taxa by altering the nutrient regime.

Effect of different organic carbon sources: Various organic carbon sources such as dextrose, glucose and sucrose (0-4%) were incorporated in the regeneration medium where sucrose rich medium was found to be superior for this plant species. Incorporation of at least one of the organic carbon sources was obligatory for regeneration and mass multiplication of plantlets. There was no regeneration in the absence of the organic carbon sources (**Table 12**). The requirements 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 organic carbon sources tested, optimum regeneration and shoot bud formation in *M. acuminata* were obtained on basal media containing sucrose (3%) where as many as 18 shoot buds were formed per subculture. The media enriched with dextrose supported fewer shoot bud formation (6-10) whereas, glucose enriched media did not support healthy regeneration.

Effect of PGRs: Different quality and quantity of PGRs marked a pronounced effect and elicit different responses in the seedling development. Inclusion of PGRs in the regeneration medium was obligatory for successful regeneration of plantlets and mass multiplication. In the absence of PGRs, there was no regeneration and cultures degenerated subsequently. Amongst the different concentrations of NAA and BA tested, a combined treatment of NAA and BA at 3 µM each supported healthy regeneration and multiple shoot formation (**Table 10**). Cultures with single treatments of BA were found to be better as compared with NAA. Cultures maintained on NAA singly supported more root formation but suppressed multiple shoot formation and leaf formation while, cultures on BA enriched media, although number of shoots were moderately high, it did not support healthy root formation. Whereas, when both NAA and BA were incorporated in combination, it supported healthy shoot formation with multiple roots. Pongener and Deb (2011a), Temjensangba and Deb (2005a) and Chen *et al* (2004) argued that the change in culture conditions and media could alter the pattern of organogenesis in orchids. Vij and Aggarwal (2003) reported that NAA favored the

development of multiple shoots/PLBs in *Vanda coerulea*. Bhadra and Hossain (2004) reported highest number of multiple shoot buds formation from nodal segment of *Micropera pallida* when medium was supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} BA. While the PGRs like BAP singly or in combination with IAA were best used for initiation of cultures and development of healthy plantlets from leaf explants of *Saccolabium papillosum* (Kaur and Vij, 2000).

Effect of AC: Besides above factors, AC at different concentrations was incorporated (0-0.5%) in the regeneration medium. Incorporation of AC in the regeneration medium facilitated the culture proliferation, induction of newer shoot buds and enlargement of pseudobulbs. While under AC control there was only about 13 shoot buds/PLBs formation in comparison to ~18 shoot buds formation on AC enriched medium (**Fig. 8**). It was observed that, when cultures were maintained on AC enriched medium, AC accelerated plant regeneration, culture proliferation and pseudobulb enlargement. The optimum response was achieved on MS medium enriched with sucrose (3%), NAA and BA (3 µM each in combination) and AC (0.3%). As in the present study, positive modification of AC in shoot induction has also been documented in *Malaxis khasiana* (Deb and Temjensangba, 2006), *Coelogyne viscose* (Vij *et. al.*, 1997a).

Effect of alternative substrata: The advanced stage PLBs/shoot buds were also maintained on the regeneration medium containing different alternative substrata such as coconut coir, foam and forest leaf litter besides agar. Within 2-3 weeks of culture on regeneration medium the advanced stage PLBs started differentiating into rooted plantlets and multiple shoot buds. For culture differentiation as well as proliferation, agar gelled medium and foam supported medium were found to be superior over other two substrata. In the present study with *M.*

acuminata, amongst the different substrata incorporated in the optimum regeneration medium, better regeneration and multiple shoot bud formation were registered on medium containing agar as gelling agent where as many as 18 shoot buds were formed. This was followed by foam (14 shoots buds), coconut coir (8) as substratum. Of the different substrata used, forest leaf litter was found to be most inferior where there was no proper regeneration of plantlets and culture proliferation. Though cultures maintained on agar gelled medium produces more secondary shoot buds/PLBs, cultures maintained on foam establish faster, plantlets are taller, leaves are dark green and culture proliferation is faster. Earlier Deb and Pongener (2010) while working with *Cymbidium aloifolium* reported that the initial response was better on agar as gelling agent as cultures established faster under this condition compared on the other substrata. However, once the cultures establish themselves on the alternative substrata especially on foam and coconut coir, they exhibited healthier growth and rapid culture proliferation compared to cultures on agar gelled medium.

During the last two decades, a number of substances viz., agarose (Johansson, 1988), alginates (Scheurich *et al*., 1980), gelrite (Pasqualetto *et al*., 1988), guar gum (Babbar *et al*., 2005; Jain *et al*., 2005), isubgol (Babbar and Jain, 1998; Jain *et al*., 1997), starch (Zimmerman *et al*., 1995; Nene *et al*., 1996), xanthan gum (Babbar and Jain, 2006) etc, have been used with reasonable success as substitutes of agar. However, these are not expected to find universal acceptance for various reasons. Alginates gel only in the presence of specific ions and therefore are not suitable substitute of agar in many circumstances, while agarose is cost prohibitive. Starch has inferior gelling ability, poor clarity as well as a metabolizable nature which can result in softening of the media. Isubgol, due to its polysaccharide nature, good gelling ability, gel clarity and resistance to enzymatic activity, has the potential to

become a universal gelling agent for plant tissue culture media, but due to its high melting point (~70°C) it needs *p*H adjustment and fast dispensing (Babbar and Jain, 2006).

Effect of substrata and their cost effectiveness

In the present study different substrata (viz., coconut coir, foam and forest leaf litter) were used as alternative to agar for culture initiation from pseudobulb segments, plant regeneration and mass multiplication. However, foam supported culture outperformed coconut coir and leaf litter supported culture in all the three stages. It was observed that the number of plantlets/shoot buds formed was higher on agar gelled medium, but cultures established faster on foam as substratum. Besides this, cultures maintained on foam supported healthier plantlet formation and rapid culture proliferation though the performance of cultures on coconut coir and leaf litter were significantly inferior compared to the others.

The hunt for the cheap substratum could be that which is/are of very low cost. In the past some efforts have been put into to use other low cost substratum to replace agar. In most of the cases success was restricted to initiation of culture only. Aggarwal *et al.* (2006) reported the use of coconut coir as substratum for seed germination of *Cymbidium pendulum* and success was restricted to germination stage only. Deb and Temjensangba (2006b), Deb and Imchen (2010), Temjensangba and Deb (2005a, 2006) could successfully use forest litter and moss as substratum in the hardening medium.

In the present study, we could successfully use coconut coir and foam disk as alternative to agar for initiation and regeneration phase. Superior regeneration of plantlets, multiple shoot bud formation was achieved on foam disk compared to other substrata. According to the estimation in the present study, the overall production cost could be

successfully reduced to ~24% compared to agar gelled culture. This distinct cost advantage is mostly due to:

(i) The agar gelled cultures demands subcultures at every 3-4 wk on fresh medium which many a times invites unwanted microbial contamination. But with the cultures on alternative substratum this problem could be ruled out as the fresh medium can be poured in the same culture vials at regular interval and only the proliferated propagules are transferred to fresh culture vials. Besides this, it cuts the manpower costs.

(ii) As most of the substances used in the present study is either the waste of households or are very cheap.

(iii) One litre of tissue culture medium with tissue culture grade agar is ~8.0 US$ where the cost of the agar is ~2.0 US$. But the cost of the foam for one litre medium is ~0.5 US$ and we could successfully recycle the foam disk up to 10 cycles. Therefore, the cost of 10 litres of medium with agar as gelling agent would be ~80 US$ but with foam as substratum it could be ~60.5 US$ only. Hence the medium cost with foam is 76% in comparison to agar gelled medium thereby reducing the production cost by~24%.

Apart from costs effectiveness, use of these substrata as in the present study does not exert pressure on nature and environment as most of the substrata are natural and renewable sources except 'foam' which is synthetic. Their increased demands can be met without any fear of exploitation of its resources and also does not pose much threat to environment on being disposed after use. It is further observed that the cultures maintained on alternative substratum establish better in the community potting mix compared to the cultures maintained on agar gelled medium as in most of the cases the roots of the regenerates adheres to the substratum as does by roots of orchids in their natural habitats.

Hardening and field trials of the regenerates

The hardening of in *vitro* raised plantlets is essential for better survival and successful establishment. In other words, the survival percentage is determined by the hardening of the plantlets. Losses of micropropagated plants after transferring to nature are attributed to low humidity, high levels of light and non-sterile condition of the *in vivo* environment (Deb and Imchen, 2010, Lavanya *et al.*, 2009; Paul, 1999). Conventionally, the tissue-raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at high light intensity. Different matrix or substrates with manipulation in salt solution were employed for hardening of different angiospermic *in vitro* raised plants by various workers viz: soilrite for *Carica papaya* (Agnihotri *et al.*, 2004), soaked cotton for *Saccharum officinarum* (Gill *et al.*, 2004) etc.

During the present investigation, the well rooted healthy plantlets were removed from the regeneration medium and transferred into culture vials containing 1/2 strength of MS salt solution supplemented with 2% (w/v) sucrose devoid of any PGRs. The cultures were then maintained for ~6-7 wk under identical culture conditions and then the hardened plants were transferred to CPM. To transplant the hardened plantlets, the CPM was prepared by mixing different substrates like charcoal pieces, chopped forest litters, coconut husk, sand and black soil (at 1:1 ratio) with a moss topping. After transferring the hardened plants to CPM they were covered with holed transparent poly bags. The potted plants were maintained in poly house (ca 70% filtered light) and fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 wk. The potted plants were left in the normal full day light conditions which were kept for about 7-8 wk before transferring to the wild. During this process plantlets turned deep green and developed pigments. About ~75% of the transplants survived to form fully developed

plants after two months of potting. Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth (Deb and Temjensangba, 2005, 2006b, 2007b; Mukherjee, 1983; Kumaria and Tandon, 1994; Temjensangba and Deb, 2005a; Pongener and Deb, 2009, 2011a, b). The acclimatized plants were transferred to Departmental botanical garden and the performances of these transplants were monitored at regular interval.

In the current investigation, the plantlets in the hardening condition were found to develop new roots. These newly developed roots get attached to the support medium with the passage of time and vigorous growth of the plantlets were observed. The roots attached themselves mostly to the charcoal pieces and moss, which strongly suggest the suitability of the material for the purpose. It was also observed that the transplanted regenerates were dark green and healthy with emergence of new roots and leaves just after one month of transfer in the potting mix.

2.4. Conclusion

During the present investigation, protocols were established for culture initiation from immature embryos of various developmental ages, foliar explants, nodal explants and pseudobulb segments from both *in vivo* and *in vitro* sources of *Malaxis acuminata*, regeneration of plantlets and mass multiplication. These techniques open new routes for *in vitro* mass multiplication of this economically important orchid species of North-East India in general and Nagaland in particular. The protocols established for culture initiation from foliar explants, nodal explants and pseudobulb segments indicates the possibility of using alternative explants. Results from the pseudobulb segments from *in vivo* source demands further research to exploit this explant source. The protocols may be used by the local

commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of Nagaland. The present investigation also offers a newer possibility of using the low costs raw materials like foam, coconut coir as alternatives to agar which will reduce the production cost considerably and will help in popularizing the plant tissue culture technique.

Chapter -3

Strobilanthes flaccidifolious Nees

3.1. Materials and Methods

Plant Collection

Strobilanthes flaccidifolious plants have been collected from Meinkong forest, Akhoya, Alichen and Longkhum area of Mokokchung district, Nagaland. The collected species were maintained in Botanical garden/experimental garden at a temperature of ~20/15°C (day/night). Regular survey was carried out in the same area at regular intervals to study their status.

Selection of Explants and Sterilization

Nodal explants: The nodal segments were collected from the tender parts of the mature plants through out the year at one month interval. 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. Subsequently explants were sterilized with aqueous solution of $HgCl_2$ (0.3%, w/v) for 5 min and subsequently rinsed 4-5 times with sterilized distilled water. Finally, the explants were dipped in ethanol (70%, v/v) for ~30 seconds and rinsed with sterilized distilled water. After sterilization, the nodal explants were soaked in sterilized water till culture.

Foliar explants: Leaves were collected from 5-6 wk old from *in vitro*/axenic culture and were used directly without sterilization.

Priming of Explants

The sterilized nodal explants and leaf were soaked in sterilized distilled water till cultured on medium. A part of the pre-soaked sterilized nodal explants were maintained on a 'Growtak Sieve' (Make: Hi-Media) containing MS medium containing sucrose (2%, w/v) and polyvinyl pryolidone (PVP) (200 mg L^{-1}) as antioxidant for 48 hr to remove the dye from the explants. The pre-soaked and primed nodal segments were used for initiation of culture.

Preparation of Substrata

Beside agar as gelling agent, three other types of substrata viz., coconut coir, forest leaf litter and polyurethane foam (hereafter called foam) were used as substrata/supporting materials as alternative to agar for regeneration and mass multiplication of plantlets. 'Foam' was collected from the local market which is generally used for preparation of mattresses. While coconut coir was extracted from the dried fruits and forest leaf litters were collected from the forest floor. All the three substrata were soaked with 'labolene' (a commercial laboratory detergent) at 1: 100 ratio (v/v) for about two hr followed by washing under running tap water till water ran clear. The substrata were air dried and stored till used. The dried coconut coir and forest leaf litter were chopped into small pieces (~0.5 cm size), while foam

was cut into disk (according to the size of culture vials). All the substrata were then autoclaved at1.05 Kg cm^{-2} pressure and 121°C for one hr before putting them in the culture vials.

Tissue Culture Media

For initiation of cultures from nodal explants full strength MS medium 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), different concentrations of PGRs like NAA and BA (0-15 µM) either singly or in combination and agar (0.8%, w/v) as gelling agent. Three different antioxidants like ascorbic acid, citric acid and PVP at different concentrations (0-400 mg L^{-1}) with increments of 100 mg was incorporated in the initiation medium. For leaf culture, MS medium were fortified with sucrose (0-4%) and agar (0.8%), PVP (200 mg L^{-1}). The medium was further fortified with different levels of PGRs like NAA, BA and Kn (0-9 µM) either singly or in combination.

The *p*H of the medium 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 cotton plugged. The medium was sterilized by autoclaving at 121°C for 20 min at a pressure of 1.05 kg cm^{-2}. Besides agar, different pre-processed substratum like coconut coir, foam and forest leaf litter were used. To which about 12 ml of the liquid media were dispensed in each test tube. The media were autoclaved at 121°C for 20 min at the pressure of 1.05 kg cm^{-2}

Plant Tissue Culture

Initiation of cultures

Nodal explants: The primed nodal segments from 'Growtak seive' were cultured on full strength MS medium fortified with different organic carbon sources (0-4%, w/v), different

antioxidants and different quality and quantity of PGRs like NAA and BA (0-15 µM) either singly or in combination and agar (0.8%, w/v) as gelling agent. About 1.0 cm long sterilized nodal segments were cultured on initiation medium and the cultures were maintained at 25±2°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*:** Leaves of *S. flaccidifolious* (~5.0 cm long) were isolated from *in vitro* stock plants (donor plants). The donor plants were raised from nodal segment cultured on MS medium containing sucrose (3%, w/v) and α-naphthalene acetic acid (NAA) (3 µM) and benzyl adenine (BA) (3 µM) in combination. The isolated leaves were soaked in sterilized distilled water till used, to wash off the dye released from the petiole. The leaf explants were cut transversely into ~1.0 cm pieces. Besides leaf segments, whole leaf was also cultured for comparative study. The leaf segments were cultured on MS medium containing sucrose (0-4%, w/v), PVP (200 mg L^{-1}) and supplemented with different concentrations of different plant growth regulators (PGRs) such as NAA, BA and kinetin (Kn) (0-9 µM) either singly or in combination.

Experimental design

A completely randomized experimental design was performed. In all the experiments, each treatment had at least 5 replicates and they were maintained at 25±2°C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} and 12/12 hr each (light/dark) photo period. All the cultures were sub-cultured at 4-5 wk interval unless mentioned otherwise. *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.

Regeneration of plantlets and culture proliferation

The shoot buds/micro shoots developed from the cultured nodal segments and foliar explants were maintained on optimum initiation medium for another two passages. The young plantlets and shoot buds were then transferred on MS medium containing different PGRs such as BA and Kn (0-9 µM) either singly or in combination. In the regeneration medium, different organic carbon sources like dextrose, glucose and sucrose (0-4%, w/v) were also incorporated. The micro shoots were separated at every sub-culture and transferred on fresh regeneration medium. A part of the shoot buds were also cultured on optimum regeneration medium (with optimum PGRs) fortified with different concentrations of AC (0-0.4%, w/v) to study the role of AC in plant regeneration and culture proliferation. Apart from agar gelled media, the cultures were also maintained on different alternate substrata such as foam, coconut coir and also forest leaf litter.

Rooting of micro shoots

Though there were 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 selected shoots were treated differently for inducing roots. One set of shoots were pulse treated with NAA (0-20 µM) for 3 hr followed by culturing on MS plain medium containing sucrose (3%, w/v) while, another set of shoot buds were cultured on MS

medium containing sucrose (3%, w/v) and NAA (0-8 µM) and maintained in normal laboratory conditions.

Hardening of the regenerated plantlets or regenerates

The well rooted plantlets were taken out from the rooting medium and transferred on medium containing ½ 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 onto 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 plants were fed with $1/10^{th}$ MS salt solution once in a week for 3-4 wk and maintained in polyshade with Ca.70% of shading sunlight and finally after two months, the plantlets were left in the normal full day light condition.

3.2. Results

Initiation of culture

Nodal explants

Seasonal effect of explants collection and priming of explants: The first objective towards the establishment of *in vitro* regeneration protocol for *S. flaccidifolious* 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

Table 14: Seasonal effect of explants collection on *in vitro* morphogenetic response of nodal explants of *Strobilanthes flaccidifolious*

Time of explants collection	% response (±SE)*	Type of response**
January	25 (1.50)	Sprouting of axillary buds, leaves crowded at the top, fewer shoot buds formation
February	25 (1.00)	As above
March	30 (0.50)	Sprouting of axillary buds from both side of the node, leaves light green, fewer shoot buds formation.
April	30 (0.75)	Greening of nodal segments, sprouting of axillary buds in both side of the node, leaves light green, plantlets healthy.
May	15 (1.50)	Release of phenolics and dye in media, Callusing of explants and in most cases tissue become necrotic
June	14 (2.00)	As above.
July	15 (1.00)	Release of phenolics and dye in media, tissue become necrotic, only swelling of node and no shoot buds formation.
August	25 (0.75)	As above but few shoot buds sprouted.
September	40 (2.00)	As above.
October	75 (2.00)	Release of dye and phenolics reduced, in most cases explants callused and fewer shoot buds formed.
November	80 (1.00)	Axillary shoot buds sprouted, multiple shoot buds formation, no release of dye, plantlets were healthy, leaves were dark green.
December	57 (1.00)	As above but fewer shoot buds formed.

* Standard error, ** On MS medium containing sucrose (3%), NAA and BA (3 μM each in combination).
Data represents the mean of five replicates.

Figure 11: Different stages of *in vitro* morphogenetic response of nodal explants of *Strobilanthes flaccidifolious* Nees. **a**. Nodal segments priming in a Growtak Seive; **b**. Nodal segment without priming cultured on initiation medium releasing dye in the medium; **c**. Sprouting of shoot buds from primed nodal segment; **d**. Multiple shoot buds formation from the cultured nodal segment.

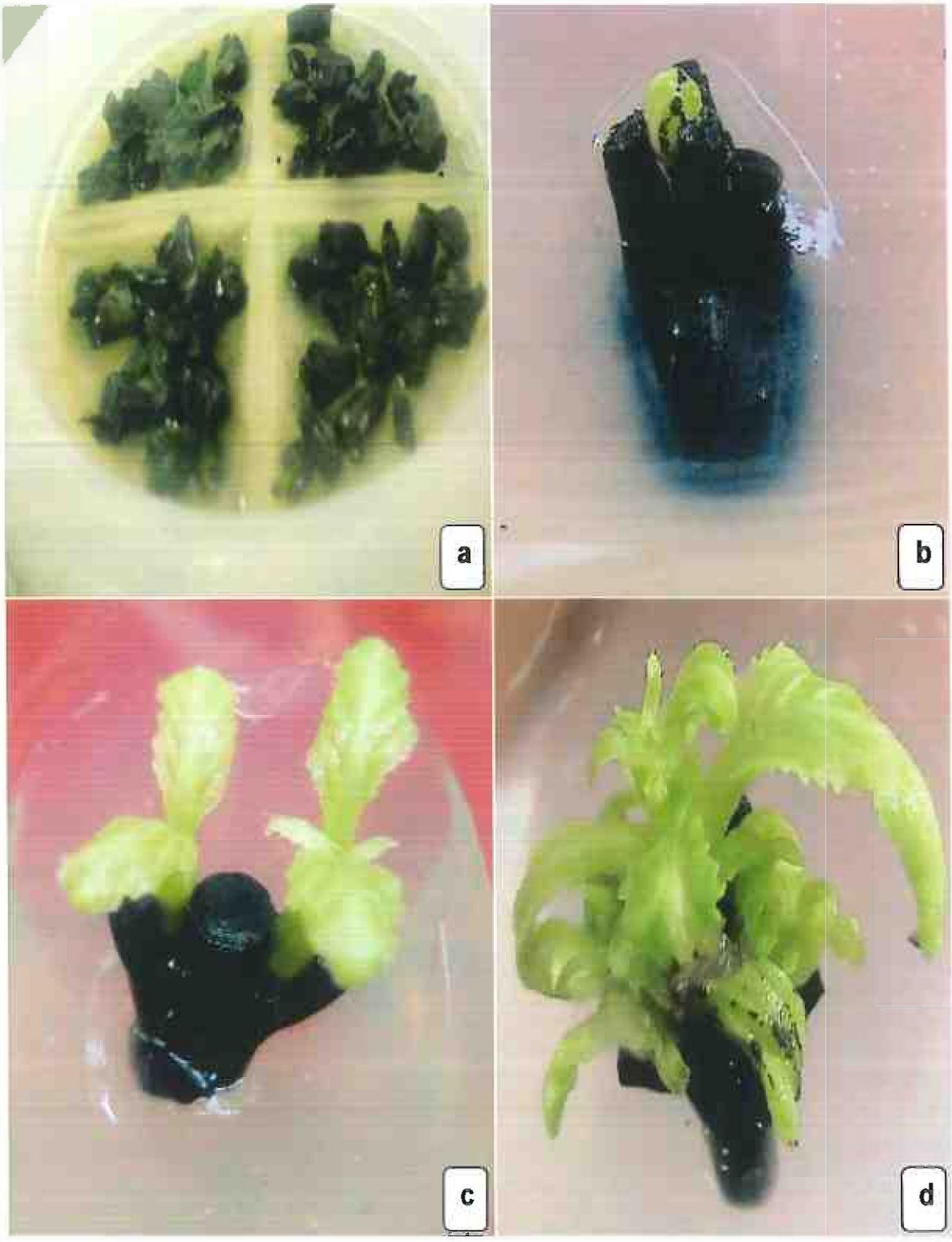

Figure-11

nodal explants in water improved the morphogenetic response over non-soaked segments. It was observed that pre-soaked nodal segments leached lesser dye in the medium in comparison to non-soaked segments. Alternatively, a part of the pre-soaked nodal segments were also maintained on a 'Growtak Seive' containing MS medium containing sucrose (2%) and PVP (200 mg L^{-1}) as antioxidant for 48 hr before transferring on initiation medium (**Fig. 11a**). During this period, the blue dye released in the liquid medium of the 'Growtak Sieve' and better morphogenetic response was achieved as compared to control and pre-soaked explants.

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. Seasonal changes greatly influenced the explants establishment. It was observed that amongst the different collection seasons studied, the nodal explants collected during May-July were least responding and tissues turned necrotic. While, explants collected during October-November responded optimally where as much as 80% nodal explants responded by sprouting the axillary buds (**Table 14**). It was observed that explants collected during May to July released dye in the culture and tissue became necrotic (**Fig. 11b**). This is probably due to the fact that during this period due to favorable rainfall and temperature, dye production is maximal. With the decrease in rainfall during October and November the leaching of dye is also decreased considerably and explants yielded morphogenetic response.

Effects of antioxidants: Under the condition employed, the primed nodal segments from mature plants were free from phenolic exudates and dye; the resident axillary buds were induced to proliferate into multiple shoot buds. As *S. flaccidifolious* is a dye yielding plant, the cultured nodal segments released excessive dye in the medium and tissue became necrotic. From necrotic explants morphogenetic response was very poor and in most of the cases

Table 15: **Effect of different antioxidants on initiation from nodal explants of *S. flaccidifolious***

Antioxidant type & Conc. (mg L^{-1})	% response	Type of response
0	0	Browning of medium and large amount of dye released, tissue became necrotic & degenerated.
PVP		
100	70 (1.0)	Browning controlled but slight release of dye.
200	*80 (1.0)*	*Healthy explants, no browning. No/less dye in the media. Axillary bud sprouted out from both the side of the node*
300	55 (2.0)	Media became brown, dye released in the media, axillary bud developed from one side of the node
400	45 (2.0)	As above
Ascorbic acid		
100	40 (2.0)	Large amount of dye released in the media, explants degenerated
200	50 (1.5)	Dye released in the media but also new leaves sprouted out
300	30 (1.0)	Moderate browning but no further response
400	25 (1.0)	As above
Citric acid		
100	45 (1.2)	Stops browning and few dye released in the media
200	35 (1.7)	As above
300	55 (2.0)	Media became brown due to release of dye in the media
400	50 (2.5)	As above

On MS medium with sucrose (3%) and NAA+ BA (3+3 µM in combination)
Data represents the mean of 5 replicates
Data collected after 45 days of culture.

Table 16: Effect of different organic carbon sources on initiation of nodal explants of *Strobilanthes flaccidifolious*

Organic Carbon Sources & Conc. (%)	No. of shoot buds formed	% response (±SE)*	Type of response**
0	0	0	No response
Dextrose			
1	2	20 ± 1.5^{e}	Shoot buds developed but stunted in growth
2	2	33 ± 2.0^{f}	Shoot bud degenerated
3	6	43 ± 3.0^{c}	Multiple shoots developed, light green in color
4	1	15 ± 1.0^{e}	Shoot buds smaller
Glucose			
1	1	15 ± 1.0^{e}	As above
2	1	18 ± 2.0^{e}	As above
3	4	40 ± 1.5^{c}	Multiple shoot buds sprouted but callusing at the base.
4	3	35 ± 1.0^{d}	Shoot buds developed but degenerated.
Sucrose			
1	3	35 ± 1.0^{d}	As above
2	8	65 ± 2.0^{b}	Shoots developed from the nodal region but smaller
3	*12*	*80 ± 1.0^{a}*	*Greening of nodal segments followed by sprouting of shoot buds at both side of the node. leaves are well expanded and green, partial swelling at the base.*
4	5	50 ± 1.0^{c}	Greening of nodal explants and shoot formation, swelling of leaves.

* Standard error. Values followed by the same *letters* are not significantly different from each other.

** On MS medium with sucrose (3%) and NAA + BA (3+3 µM in combination)

Data represents the mean of 5 replicates each

Data scored after 6 wk of culture

explants degenerated subsequently. For inducing morphogenetic response from the nodal explants, it was prerequisite to stop the leaching of dye and other phenolic compounds in the medium. Various antioxidants like PVP, ascorbic acid and citric acid at various concentrations (0-400 mg L^{-1}) were incorporated in the initiation medium to stop/reducing the exudation of dye and phenolic compounds (**Table 15**). Both ascorbic acid and citric acid across the concentrations tested though stopped the exudation effectively, but did not support morphogenetic response. Under the conditions provided, PVP at a concentration of200 mg L^{-1} reduced the leaching of dye moderately and also supported the morphogenetic response. Under optimum condition ~80% nodal explants responded positively (**Table 15**).

- ***Effects of organic carbon sources***: Incorporation of at least one organic carbon source 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, ~40% of the explants invoked meristematic loci on basal medium enriched with 3% glucose where as many as 4 shoot buds developed from each node (**Table 16**). But when dextrose used as organic carbon, under optimum concentration (3%) ~43% explants responded positively where average 6 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 ~80% nodal segments after 6 wk of culture.

Table 17: **Effects of PGRs on *in vitro* morphogenetic response of nodal segments of *Strobilanthes flaccidifolious*.**

PGRs Conc. (µM)* NAA	BA	% response (±SE)**	No. of shoot buds formed/explants	Type of response***
0	0	0	0	Greening of explants but degenerated
-	3	43±2.0^{c}	14^{a}	Greening of explants followed by sprouting of axillary buds, multiple shoot buds formation, leaves green.
-	6	44±3.0^{c}	6^{b}	Multiple shoot buds sprouted but callusing at the base.
-	9	43±2.5^{c}	4^{c}	As above.
-	12	20±1.5^{e}	1^{d}	Shoot buds formed but degenerated.
-	15	0	0	No response.
3	0	67±0.5^{b}	2^{d}	Swelling of explants at nodal zone and fewer shoot buds formed.
6	0	40±1.0^{c}	2^{d}	As above.
9	0	0	1^{d}	Swelling of nodal explants but degenerated subsequently.
15	0	25±2.0^{e}	1^{d}	As above.
3	**3**	**80**±1.0^{a}	**12**^{a}	**Greening of nodal segments followed by sprouting of shoot buds at both side of the node, leaves are well expanded and green, partial swelling at the base.**
3	6	57±2.5^{b}	2^{d}	Greening of nodal explants and shoot formation, swelling ofleaves.
3	9	33±2.0^{d}	1^{d}	Greening of nodal explants followed by callusing of explants.

* Only the significant treatments are computed;

** Standard error, In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls).

*** On MS medium containing sucrose (3%, w/v);

Data represents the mean of five replicates;

Data scored after 5 wk of culture initiation.

Table 18: Effect of quality and quantity of PGRs on inducing morphogenetic response in foliar explants of *Strobilanthes flaccidifolious*#.

PGRs Conc. (µM)*			Time for initial response (days)	% response (±SE)**	Morphogenetic pathway***	No. of shoot buds formed /explants
NAA	BA	KN				
0	0	0	-	-	-	-
3	-	-	15	25±0.50^{g}	Ca-Rt	1
6	-	-	7	66±1.00^{c}	Rt	1
9	-	-	7	66±0.75^{c}	Rt	1
-	3	-	7	70±1.50^{b}	Ca	3
-	*6*	-	*10*	*75±0.75*^{a}	*Ca-Sb*	*9*
-	9	-	7	70±2.50^{b}	Ca-Sb-Rt	6
-	-	3	15	60±2.00^{d}	Sb	4
-	-	6	20	50±1.50^{e}	Sb	3
-	-	9	20	50±1.75^{e}	-	-
3	3	-	12	25±1.00^{g}	Ca	2
3	6	-	10	75±1.50^{a}	Ca-Rt	2
3	9	-	8	65±2.50^{c}	Ca	-
6	3	-	10	60±3.00^{d}	Ca	-
6	6	-	7	60±2.50^{d}	Ca	-
6	9	-	10	50±1.00^{e}	Ca	1
9	6	-	10	63±2.50^{d}	Ca	-
9	9	-	20	48±1.00^{f}	Ca-Rt	1
3	-	3	25	45±2.00^{f}	Ca	2
3	-	6	30	55±1.50^{e}	Ca	2
6	-	6	30	20±1.00^{g}	Ca	1

On MS medium containing sucrose (3%, w/v), PVP (200 mg L^{-1})

* Only the significant treatments are computed.

** Standard error, in the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls).

*** Ca: Callus, Sb: Shoot buds, Rt: Roots.

Data represents the mean of five replicates, Data scored after 5 wk of culture initiation.

Figure 12: Different stages of *in vitro* morphogenetic response of foliar explants of *Strobilanthes flaccidifolious*. **a**. Cultured leaf explants showing swelling and curling of leaf segments; **b**. Callusing and direct shoot buds formation from the cultured leaf explants; **c**. Multiple shoot buds formation from the cultured leaf explants.

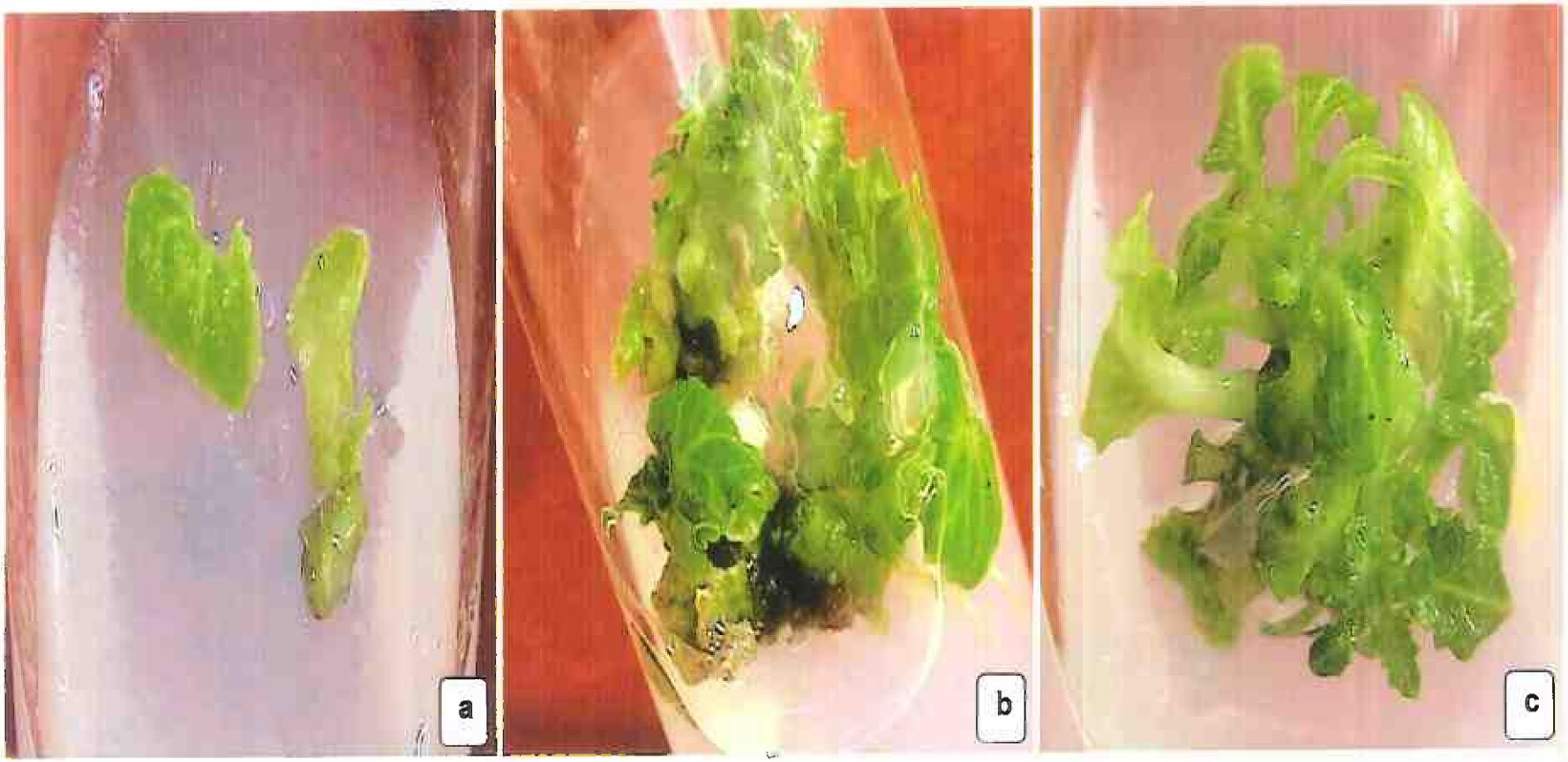

Figure - 12

Effects of PGRs: Swelling of the axillary buds was observed within a week followed by differentiated into multiple shoot buds/micro shoots formation within 5-6 wk (**Fig. 11c**). Presence of PGRs in the medium was necessary for morphogenetic response. In the absence there was only greening of the explants but degenerated without morphogenesis. Of the two PGRs incorporated at differential concentrations, NAA singly did not impact impressive morphogenetic response and in most of the cases explants callused, while BA singly at a concentration of 3 µM supported maximum number of shoot bud formation. But when NAA and BA used in combination, highest number of nodal explants responded positively. About 80% of the nodal explants responded positively on medium containing sucrose (3%) and NAA + BA (3 µM each in combination) where as many as 12 shoot buds/micro shoots formed without callus formation (**Table 17, Fig. 11d**). At higher concentrations of combined treatments explants callused in most of the cases.

Foliar explants

Soaking of leaf explants in water after excising from the *in vitro* raised cultures improved the morphogenetic response over non-soaked explants. It was observed that pre-soaked leaf explants leached dye in the water and promoted healthy culture initiation. While, the leaf explants which were not soaked released dye and phenolic compounds in the medium, became necrotic and degenerated subsequently. Under the condition employed, the primed leaf explants were free from dye and phenolic exudates; the meristemetic loci were invoked followed by shoot bud formation. Swelling of foliar segments was observed within 7 days of culture from the cut ends followed by either shoot buds formation or callus formation within 3-4 wk (**Table 18, Fig. 12a**). The highest percentage of cultivated leaf segments forming

protuberances/shoot buds was 75% and highest mean number of protuberance regenerating buds per segment was 9 (**Table 18, Fig. 12b**).

Incorporation of sucrose in the medium was prerequisite for induction of morphogenetic response. On medium free of sucrose, leaf explants failed to respond and degenerated subsequently. Of the different concentration of sucrose tested, better morphogenetic response was achieved on medium fortified with sucrose (3%) (data not presented). Medium containing higher concentration of sucrose, explants turned brown while, at lower concentration fewer shoot buds formed. For morphogenetic induction, three different PGRs were incorporated at different concentrations. All the concentrations of NAA either singly or in combination with BA and Kn supported callus induction while both BA and Kn supported either shoot bud formation or callus formation. Lower concentrations of BA supported callus formation while at higher concentration of BA shoot bud induction was recorded. At a concentration of 6 µM BA, ~75% of explants responded positively where as many as 9 shoot buds/micro shoot formed (**Table 18**) accompanied by callus formation. For morphogenetic response, leaf segments as well as whole leaf was tested. In the present study, morphogenetic response was initiated from the cut ends of leaf segments while response was comparatively poorer in whole leaf. The shoot buds so formed converted into plantlets and also formed multiple shoots when maintained for another two passages on optimum initiation medium (**Fig. 12c**).

Plant regeneration and culture proliferation

The meristematic loci/shoot buds/micro shoots developed from nodal segments and foliar explants on initiation medium were maintained for another two passages for culture differentiation and proliferation. The micro shoots are then maintained on MS medium

Table 19: Effects of different quality and quantity of organic carbon sources on regeneration of plantlets and culture proliferation of *Strobilanthes flaccidifolious*

Organic carbon Source & conc(%)	Height of plantlet (cm)	No. of leaves	No. of shoots	No. of roots	Type of response*
0	0	0	0	0	No response
Dextrose					
1	1.8	5	6	1	Leaves dark green, few curled.
2	1.9	6	5	1	Leaves light to dark green, unhealthy
3	1.8	5	1	1	Leaves light to dark green, broader leaves
4	1.9	5	3	5	Leaves broad, dark green, opened
Glucose					
1	1.6	5	1	1	Smallerleaves,opened
2	1.9	6	2	1	Leaves dark green, opened
3	1.8	6	6	1	Shoots small, leaves small
4	2.7	7	2	4	Leaves dark green, opened.
Sucrose					
1	2.6	6	5	1	Leaves light green, opened
2	3.4	7	3	2	Leaves small, unhealthy
3	4.5	8	7	10	Leaves dark green with broad leaves
4	2.9	5	3	2	Leaves small, unhealthy and degenerated gradually

*On MS medium with Kn (3 μM)

Data represents the mean of 5 replicates

Data collected after 7 wk of culture

containing different organic carbon sources (dextrose, glucose and sucrose) at different concentrations (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.

Effects of quality and quantity of organic carbon sources: It was found that presence of at least one of the organic carbon sources in the regeneration medium was prerequisite by the fact that in the absence of any of the organic sources there was no regeneration of plants and cultures degenerated within 2-3 week after culture. Presence of dextrose at a concentration of 1% exhibited better response where as many as 6 shoot buds with an average 5 leaves per plant formed after ~7 wk of culture, but in this concentration only one root per plantlets developed. But higher concentration (4%) though number of shoots was lesser (3 number) but it resulted in more roots (5 numbers per plant). When glucose was added as carbon source, at lower concentrations (1 and 2%) did not support healthy plant regeneration and multiple shoot buds formation. But at higher concentration (3%) supported more numbers of shoot bud formation where as many as 6 shoots developed. But presence of sucrose in the regeneration medium out performed both dextrose and glucose (**Table 19**). Sucrose across the concentrations was found to be superior over other sources. Under optimum concentration of sucrose (3%) produced ~7 shoots and there was 10 roots per plants.

Effet of different cytokinins: For regeneration of plantlets, incorporation of one of the PGRs was obligatory. In medium freed of any PGRs, cultures exhibited stunted growth and degenerated. Presence of BA across the concentrations either singly or in combination did not promote healthy plantlet regeneration or culture proliferation and plantlets exhibited stunted growth. Singly treatment of BA (3 µM) supported mean 4.2 numbers of shoot bud formation

Table 20: Effects of different cytokinins on plantlets regeneration and culture proliferation of *Strobilanthes flaccidifolious*

PGRs Conc. (µM)# BA	Kn	No. of shoot buds formed/explant	Mean plantlet height (cm.)*	Type of response**
0	0	0	0	Growth stunted and degenerated
3	0	4.2[b]	3.2±0.1[c]	Plantlets with dark green small leaves but plantlets etiolated
6	0	3.1[c]	4.0±0.1[b]	Plantlets slightly light green and not healthy
9	0	2.2[d]	3.6±0.2[b]	Small leaves and slight callusing at the base
0	*3*	*7.3[a]*	*4.5±0.2[a]*	*Healthy plantlets with dark green and broad leaves, plantlets with few roots*
0	6	3.2[c]	3.0±0.3[c]	Poor plant growth, yellowish-green leave
0	9	2.1[d]	2.9±0.2[c]	Stunted plant growth
3	3	5.2[b]	4.3±0.1[a]	Dark green leaves, stunted growth with fewer roots
3	6	3.3[c]	3.5±0.3[b]	As above
3	9	3.2[c]	3.6±0.2[b]	As above
6	3	3.4[c]	3.0±0.2[c]	Stunted plant growth and leaves light green
6	6	2.3[d]	2.7±0.2[d]	As above
6	9	3.2[c]	3.2±0.5[c]	Many small leaves crowded at top, leaves light green
9	3	2.5[d]	2.7±0.1[d]	Broad green leaves, healthy plantlets
9	6	3.4[c]	2.5±0.2[d]	As above
9	9	2.3[d]	2.9±0.2[c]	Leaves light green and plantlets unhealthy

Only the significant treatments are computed.
* Standard error, in the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls).
** On MS medium containing sucrose (3%, w/v);
Data represents the mean of three replicates.
Data scored after 8 wk of culture on regeneration medium.

Figure 13: Plantlets regeneration and multiple shoot buds formation of *Strobilanthes flaccidifolious* on regeneration medium containing different substrata. **a**. Multiple shoot buds formed on agar gelled regeneration medium; **b**. Multiple shoot buds formed on regeneration medium containing foam as substratum; **c**. Multiple shoot buds formed on regeneration medium containing coconut coir as substratum; **d**. Multiple shoot buds formed on regeneration medium containing forest leaf litter as substratum.

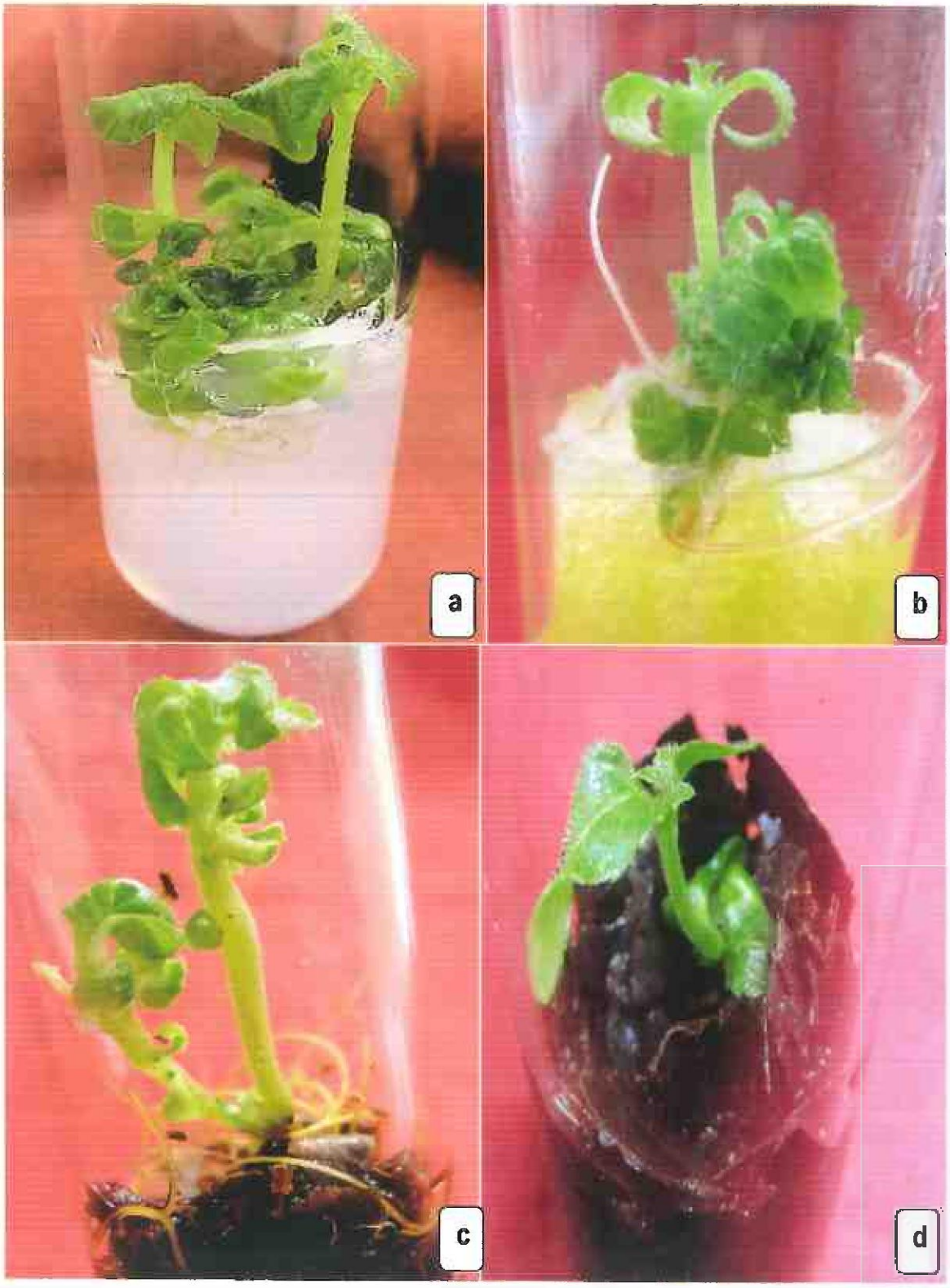

Figure-13

Figure 14: Effect of Activated charcoal on regeneration of plantlets of *Strobilanthes flaccidifolious.*

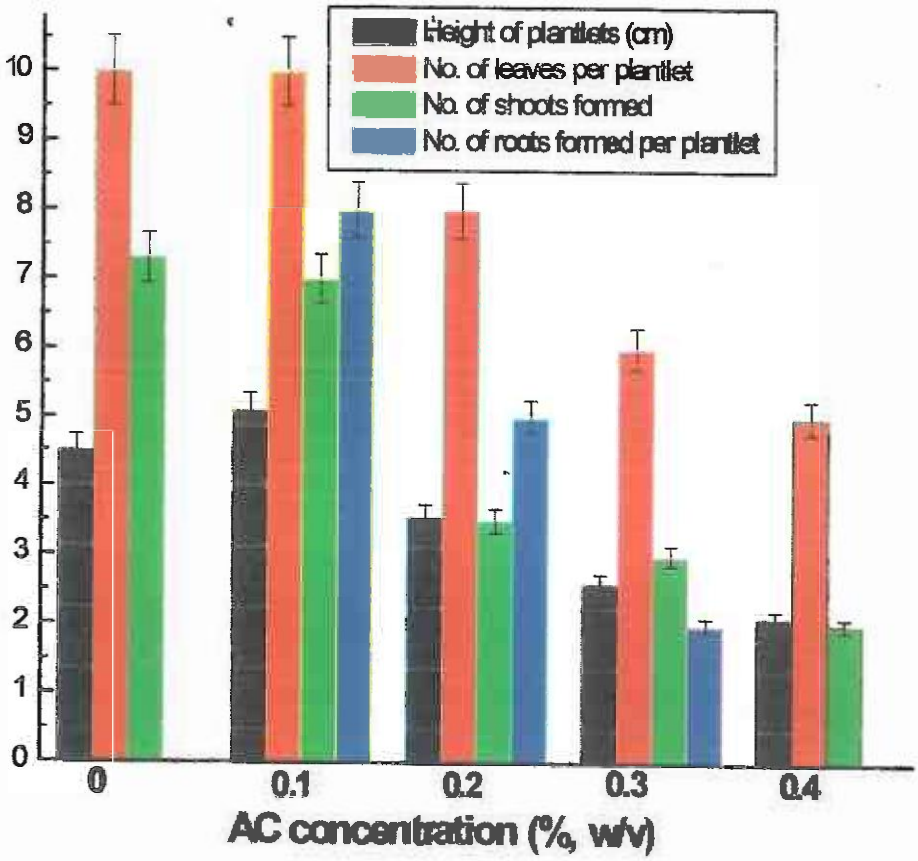

Figure 14: Effect of activated charcoal on regeneration of plantlets of *Strobilanthes flaccidifolious.*

Table 21: Regeneration of *Strobilanthes flaccidifolius* on different alternative substrata

Type of substratum	Mean height of plantlets (cm)	No. of leaves per plantlet	No. of shoot per plantlet	No. of roots per plantlet	Type of response
Agar	4.5	8	7	10	Healthy plantlet leaves many, dark green, broad. Roots long and healthy
Cocoaut coir	1.52	5	2	1	Leaves dark green, small. Plantlet degenerated gradually.
Foam	1.95	7	5	3	Leaves dark green, opened, shoots small Leaves small, dark green, opened.
Forest litter	1.48	5	-	-	Plantlets unhealthy & degenerated gradually.

*On MS medium with sucrose (3%) and PGR- Kn (3 μM)

Data represents the mean of 5 replicates each

Data collected after 35 days of culture

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 3 µM each of both BA and Kn formed 5.2 shoot buds and mean plant height was 4.3 cm. But incorporation of Kn singly proved to be optimum under the given conditions for shoot proliferation and plant growth. A mean of 7.3 shoot buds/micro shoots of ~4.5 cm height were formed on medium containing Kn (3 µM) (**Table 20, Fig. 13a**).

Effect of AC on plant regeneration. In one set of regeneration medium with optimum PGRs, AC was incorporated to study its effect on plant regeneration and plant morphology. It was observed that incorporation of AC had very little or no effect on culture proliferation and plant growth. Though AC had very little effect on multiple shoot formation but, on AC rich medium plantlets developed roots which was absent in AC control medium. As many as 8 roots per plant formed on medium containing 0.1% (w/v) AC. While at higher concentrations of AC culture proliferation as well as culture growth inhibited (**Fig. 14**).

Effects of alternative substrata on plant regeneration and culture proliferation. As described with *M. acuminata*, different substrata like coconut coir, foam and forest litter were also used against agar in the regeneration medium. When compared the regenerative performance on different substrata including agar, it was found that agar as gelling agent performed better compared to other substrata. On agar gelled medium, there was as many as 7 plants with about 10 roots per plant and plant height was ~4.5 cm. But on foam as substratum, there was only 5 shoots with 3 roots per plant and plant height was ~2.0 cm (**Table 21**). On the other hand, cultures maintained on coconut coir supported only 2 plantlets formation while, there was no regeneration on forest leaf litter and cultures degenerated. When

Figure 15: Rooting, hardening of rooted plants of *Strobilanthes flaccidifolious* and transplanting to community potting mix. **a**. Regenerated plantlet transferred for rooting on medium enriched with NAA (3 μM); **b**. Plantlet transferred for rooting on plain medium after pulse treatment with NAA (10 μM); **c**. A rooted plant on hardening condition; **d**. Hardened plant transferred to community potting mix.

Figure-15

Table 22: **NAA stimulated *in vitro* rooting of micro shoots of *Strobilanthes flaccidifolious*.**

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	14[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 five replicates.
Data scored after 4 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).

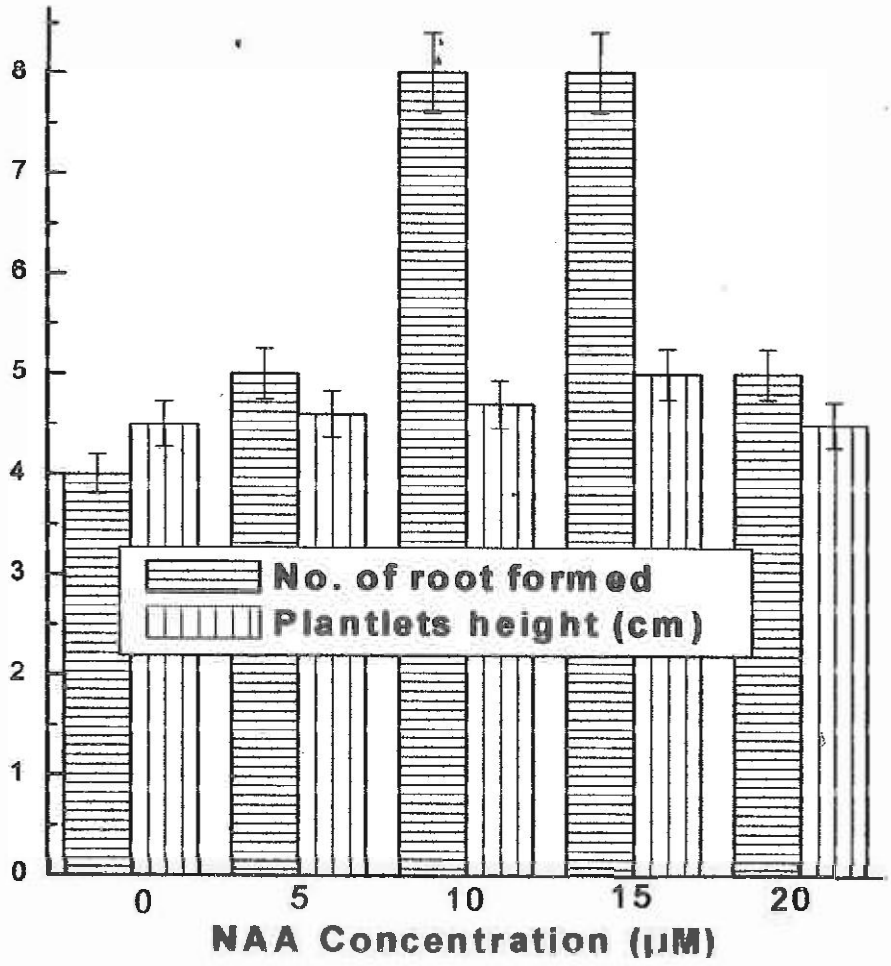

Figure 16: Effect of plus treatment with NAA on inducing roots of *S. flacciidifolious* micro shoots.

compared the plant morphology on the four different substrata, it was observed that plants cultured on agar gelled medium and foam disk were healthy and dark green while cultures on coconut coir and leaflitters were not healthy and pale green in color (**Fig. 13a, b, c, d**).

Rooting of micro shoots

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 differently for inducing roots. In general, pulse treatment with NAA was found to be inferior over incorporation of NAA in the medium (**Fig. 15a and 15b**). Of the different concentration of NAA used for inducing roots, a concentration of 3 µM supported maximum root growth where as many as 14 roots per plant developed after 4 wk of culture (**Table 22, Fig. 15a**). At lower concentrations roots were shorter and plantlets were etiolated while, at higher concentrations, roots formation was impaired accompanied by swelling of plants as well as roots.

In comparison to above, the pulse treatment of micro shoots with NAA, roots formation as well as shoot growth was poor (**Fig. 15b, 16**). Under optimum condition, only 8 roots were formed after ~4 wk of culture against 14 roots when NAA was incorporated in the medium (**Table 22**). Amongst the different pulse treatments given for inducing roots, pulse treatment with NAA at concentrations of 10 and 15 µM were equally effective for inducing roots and vertical increase in plant height.

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 (**Fig. 15c**). 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 onto plastic pots containing a mixture of soil, sand, decayed wood powder at 1:1 ratio with a moss topping (**Fig. 15d**). The pots were covered with holed transparent polybags and watered at week interval for two months. The plants were fed with $1/10^{th}$ MS salt solution once in a week for 3-4 wk and maintained in polyshade with Ca.70% of shading sunlight and finally after two months, the plantlets were left in the normal full day light condition. About 200 plants were tested for survival and about 70% survival was registered after two months of transfer.

3.3 Discussion

Seasonal effect of explants collection and priming: *S. flaccidifolious* is a natural dye yielding plant and releases the dye instantly from the cut ends. For successful culture initiation from both nodal explants as well as foliar explants, it was necessary to stop/reduce the exudation of dye and phenolic compounds in the medium. The first objective towards the establishment of *in vitro* regeneration protocol for *S. flaccidifolious* was to optimize the time of nodal explants collection from the field grown plants. The nodal segments were soaked in sterilized 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 dye in the medium in comparison to non-soaked segments. Earlier Lakshmanan *et. al.* (2006) in sugar cane leaf culture observed that soaking of leaf explants after excision promoted morphogenetic response *in vitro*.

A part of the pre-soaked nodal explants were also primed by maintaining on a 'Growtak Seive' containing MS medium with sucrose (2%) and PVP (200 mg L^{-1}) as

antioxidant for 48 hr before transferring on initiation medium. During this priming period, the blue dye released in the liquid medium of the 'Growtak Sieve' and better morphogenetic response was achieved as compared to control and pre-soaked explants. Earlier Deb and Tandon (2004b) treated the apical dome section at 4°C for 24 hr on mMS medium containing 0.4% AC before transferring to initiation medium for raising successful embryogenic culture of *Pinus kesiya*.

Round the year, the nodal explants were collected at one month interval starting from January till December. In the present study with *S. flaccidifolious*, 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 May-July were least responding and tissues turned necrotic. While, explants collected during October-November responded optimally where as much as 80% nodal explants responded by sprouting the axillary buds while explants collected during May to July released dye in the culture and tissue became necrotic and cultures degenerated in most of the cases. This is probably due to the fact that during this period due to favorable rainfall and temperature, dye production is maximal. With the decrease in rainfall during October and November the leaching of dye is also decreased considerably and explants yielded morphogenetic response. 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 were 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 (Bhat and Chandel, 1991). In the present study, release of dye and browning of medium was one of the bottlenecks for successful establishment of culture from both the explants sources in general and nodal explants in particular. The release of dye and browning of medium could be prevented by incorporating antioxidant like PVP (200 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 both nodal explants as well as foliar 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: Swelling of the axillary buds was observed within a week followed by differentiated into multiple shoot buds/micro shoots formation in 3-4 wk from nodal segments while, within one wk of culture the cultured foliar explants started swelling. 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). 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 two PGRs incorporated at differential concentrations for culturing nodal explants, NAA singly did not impact impressive morphogenetic response, while BA singly at a concentration of 3 µM supported maximum number of shoot buds formation. But when NAA and BA used in combination, highest number of nodal explants responded positively. About 80% of the nodal explants responded positively on medium containing sucrose (3%) and NAA + BA (3 µMeach in combination) where as many as 12 shoot buds/micro shoots formed without callus formation. 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).

While working with the foliar explants, it was observed that uptake of BA by *S. flaccidifolious* leaves cultured on MS medium promoted partial callus followed by shoot bud formation. It is important to highlight that mother plants which provided the leaves for the present study were cultured on MS medium containing BA and NAA. Thus, in the absence of NAA in the initiation medium, BA singly could promote shoot bud formation and this might be related to absorption of NAA from medium and accumulation in the mother plants. As a result, when NAA used either singly or in combination with BA or Kn promoted callusing. 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 were maintained for another two passages. The micro shoots are then maintained on MS medium containing different organic carbon sources and different PGRs.

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 4 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 7 shoot buds with an average of 8 roots were formed on medium containing Kn (3 µM) where average plant height was ~4.5 cm (**Table 20**). 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).

Effect of AC on plant regeneration: While working with woody species, incorporation of AC in the culture medium promotes morphogenetic response. It is believed that AC in the medium can absorbs growth inhibitors/retardants, phenolic substances from the explants resulting in early morphogenetic response (Bonga and Aderkas, 1992). In the present study, AC was added in the regeneration medium at different concentrations (0-0.4%). In general, incorporation of AC in the regeneration medium had little or no impact on culture proliferation and plant growth. It was observed that on AC rich medium plantlets developed roots which is absent in AC control medium. As many as 8 roots per plant formed on medium

containing 0.1% (w/v) AC. While at higher concentrations of AC, culture proliferation as well as culture growth inhibited. But the positive effect of AC is described in *Coelogyne viscose* (Vij *et. al.*, 1997b).

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. In general, pulse treatment with NAA was found to be inferior over incorporation of NAA in the medium. Of the different concentration of NAA used for inducing roots, a concentration of 3 µM supported maximum root growth where as many as 14 roots per plant developed after 4 wk of culture. At lower concentrations, roots were shorter and plantlets were etiolated while, at higher concentrations, roots formation was impaired accompanied by swelling of plants as well as roots.

In comparison to above, the pulse treatment of micro shoots with NAA, roots formation as well as shoot growth was poor (**Fig. 2**). Under optimum condition, only 8 roots were formed after ~4 wk of culture against 14 roots when NAA incorporated in the medium. Amongst the different pulse treatments given for inducing roots, pulse treatment with NAA concentrations of 10 and 15 µM were equally effective for inducing roots and vertical increase in plant height. Auxin pulse treatment, as a method to replace the induction phase and thus, to simplify and improve the rooting system, has been described in several species, including apple (Moncousin *et. al.*, 1992), *Quercus suber* (Manzanera and Pardos, 1990), olive (Peire *et. al.*, 2007). 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.

Effects of alternative substrata. The shoot buds/micro shoots developed on initiation medium were also maintained on three different substrata as alternative to agar. In general, agar as gelling agent was found to be superior over other substrata. Though the alternative substrata were found to be inferior, foam as substrata was found to be competitive with agar. Earlier reports suggest that these substrata could be successfully used as alternative to agar in orchid (Deb and Pongener, 2010, Pongener and Deb, 2011b). Present study was aimed to extend their use as alternative to agar in non-orchid species and could be used with partial success. The advantages and cost effectiveness of using these substrata is described in the previous chapter with *Malaxis acuminata*.

3.4. Conclusion

During the present investigation, protocols were established for culture initiation from nodal explants from *in vivo* source, foliar explants of *in vitro* source. These techniques open

new routes for *in vitro* mass multiplication of this economically important species of North-East India in general and Nagaland in particular. The protocols established for culture initiation from 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 handloom industry. During the present investigation, some alternative substrata could be used as alternative to agar with partial success for reducing production cost. Future research warrants on utility of these and other low cost materials as alternative substrata so that the production cost could be reduced substantially.

Chapter-4

Summary

The state Nagaland is located in North Eastern region of India and lies between 25°6′-27°4′ of latitudes and 93°20′-95°15′ E longitude with geographical area of about 16, 57,900 hectares and forest covers an area of 8, 62,930 hectares. The state has rich plant genetic resources and represented by over 2500 species belonging to near 963 genera and 186 families of angiosperm flora including many endemic plant species of the region. The natural vegetation of the region is under great threat and 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. During the current study, I have worked with two economically important plant species viz., ***Malaxis acuminata*** D. Don (Orchidaceae) and ***Strobilanthes flaccidifolious*** Nees (Acanthaceae) of Nagaland, India for their *in vitro* propagation and establishment in the natural habitats. The cultures were

initiated from different explants sources like immature embryos/seeds, nodal explants, foliar explants and pseudobulb segments of the two selected plant species.

1. *Malaxis acuminata* D. Don (Orchidaceae)

Cultures were initiated from immature embryos/seeds of 7-8 weeks after pollination (WAP). The seed cultured from green pod of 7 WAP exhibited germination of ~85% after 135 days (~19 wk) of culture initiation. Nodular swelling of embryos/seeds followed by bursting out of testa was observed after 8 wk of culture. Seeds younger than 6 WAP either did not germinate or exhibited delayed germination while, seed age >9 WAP, germination frequency was comparatively lower.

Germination rate of ~85% was achieved on MS medium against 65% on Mitra *et al* medium containing sucrose (3%, w/v). Amongst the different quality and quantity of organic carbon used, sucrose at a concentration of 3% supported optimum germination and seeds germinated early. At lower concentration of sucrose, fewer PLBs were formed while at higher concentration, there was delayed germination. Both dextrose and glucose could not support healthy germination.

Amongst the different levels of PGRs used for non-symbiotic seed germination, MS medium containing NAA (4 µM) singly supported optimum germination (~85%) after 55 days of culture and formation of healthy PLBs after 135 days of culture. At lower concentration of NAA germination delayed while at higher concentrations germination rate was poorer. The lone treatment of BA across the concentrations and in combination with NAA delayed germination and formed fewer PLBs. The cultures were maintained under full light condition (40 µmol m^{-2} s^{-1} light intensity) at 12/12 hr (D/L) photoperiod and at 25±2°C

supported better germination followed by cultures maintained in diffused light condition (20 μ mlo m^{-2} s^{-1}).

Cultures were also initiated from the foliar explants (~5-6 wk old), nodal explants and pseudobulb explants from *in vitro* source. After 4-5 wk of culture initiation, meristematic activity was invoked at the basal ends of the cultured foliar explants. About 65% of cultured explants responded positively after 28 days of culture initiation on MS medium containing sucrose (3%) and NAA + BA (3 and 6 μM respectively in combination) where as many as 26 meristemoids/shoot buds invoked and formed shoot buds/PLBs.

Sprouting of the shoot buds/PLBs from the nodal region of the segments was initiated within 3 wk of culture where as many as 18 shoot buds/PLBs developed at a single node on MS medium supplemented with sucrose (3%) and NAA + BA (3+3 μM in combinations). About 90% explants responded positively with the sprouting of shoot buds/PLBs from the nodal regions.

Cultures were also initiated from pseudobulb segments both from *in vitro* as well as from *in vivo* sources. About 15% explants from *in vivo* source responded positively with an average of 2-3 shoot buds formed per segment on medium enriched with sucrose (3%) and NAA (6 μM) and BA (6 μM) in combination. In comparison to pseudobulb from *in vivo* source, pseudobulb harvested from *in vitro* source exhibited a contrasting response. About 95% explants responded positively with about 11 shoot buds formed per segment on medium containing sucrose (3%), NAA and BA (6 μMeach in combination). Incorporation of AC in the medium did not support healthy morphogenetic response. On AC control medium there was as many as 11 shoot bud/PLBs formation while on AC enriched medium under optimum condition (0.3%) there were only 8 shoot buds formation. Though incorporation of AC in the

medium did not support optimum shoot buds/PLBs formation but it supported invocation of roots in the shoot buds. As many as 5 roots developed per plantlets while there was no root formation in AC control medium.

Besides agar gelled medium, the pseudobulb segments from *in vitro* source were also maintained on three other substrata viz., foam, coconut coir and forest leaf litter in two different basal media i.e., MS and Knudson 'C' media. In both the media, agar gelled media supported better morphogenetic response followed by foam as substrata. While, coconut coir and forest litter as substrata did not support healthy response.

The shoot buds/PLBs formed from asymbiotic seed germination, foliar explants, nodal explants and pseudobulb explants were cultured on two different basal media viz., MS and Mitra *et al* media with different adjuncts for regeneration of plantlets and culture proliferation. Better growth, development and differentiation into rooted plantlets were recorded on MS basal medium. Further studies with different strengths of MS medium ($1/4^{th}$, ½, $3/4^{th}$ and full strength) were conducted on regeneration of plantlets and culture proliferation and it was found that full strength MS medium supported the healthy regeneration and culture proliferation compared to other strengths. The basal medium was supplemented with different quality and quantity of different organic carbon sources (dextrose, glucose and sucrose at a concentration of0-4%) and it was found that the optimum regeneration and shoot buds formation were obtained on basal medium containing sucrose (3%).

When cultures were maintained on AC enriched medium, it accelerated plant regeneration, culture proliferation and pseudobulb enlargement and also root formation. The optimum response was achieved on MS medium enriched with sucrose (3%), NAA and BA (3

µMeach in combination) and AC (0.3%) where average 4.5 leaves, 4 roots per plants and 18 shoot buds formed per subculture.

Besides agar, the advanced stage PLBs/shoot buds were also maintained on the regeneration medium containing different alternate substrata such as coconut coir, foam and forest leaf litter. Amongst the different alternate substrata used, better regeneration and multiple shoot buds formation were registered on medium containing foam disk as substratum and agar gelled medium where as many as 18 shoot buds developed on agar gelled medium against 14 shoot buds on foam disk. On media with coconut coir and forest leaf litter as substrata, the number of shoot buds produced was much lower. Besides this, cultures maintained on these two conditions required prolonged period for differentiation. Though cultures maintained on agar gelled medium produces more secondary shoot buds/PLBs, cultures maintained on foam establish faster, plantlets were taller, leaves are dark green and culture proliferation was faster.

The well rooted plantlets on the regeneration medium were maintained for about 2-3 passages for further growth followed by transferring them in the hardening medium. The well rooted healthy plantlets were hardened for 6-7 wk in normal laboratory condition in culture vials containing 1/2 strength MS medium supplemented with sucrose (2%) but freed from any PGRs. The hardened plants were transferred to community potting mix containing a mixture of charcoal pieces, chopped forest litters, coconut husk, sand and black soil (at 1:1 ratio) with a moss topping. The transplants were covered with holed transparent poly bags and maintained in poly house (ca 70% filtered light). The plants were fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 wk. The potted plants were left in the normal full day

light conditions which were kept for about 7-8 wk before transferring to the wild. About 75% of the transplants survived to form fully developed plants after two months of potting.

2. *Strobilanthes flaccidifolious* Nees (Acanthaceae)

The first objective towards the establishment of *in vitro* regeneration protocol for *S. flaccidifolious* was to optimize the time of nodal explants collection from the field grown plants. The nodal segments were collected round the year at one month interval starting from January till December. Of the different collection seasons studied, nodal explants collected during May-July was least responding and tissues turned necrotic. While, explants collected during October-November responded optimally where as much as 80% nodal explants responded by sprouting the axillary buds. The sterilized nodal segments were primed by maintaining on a 'Growtak Seive' containing MS liquid medium, sucrose (2%) and PVP (200 mg L^{-1}) as antioxidant for 48 hr before transferring on initiation medium.

About 80% of primed nodal explants responded positively on MS medium enriched with sucrose (3%, w/v), NAA and BA (3 µM each in combination) and formed shoot buds. Of the two PGRs incorporated at differential concentrations, NAA singly did not impact impressive morphogenetic response and in most of the cases explants callused, while BA singly at a concentration of 3 µM supported maximum number of shoot buds formation. But when NAA and BA used in combination, highest number of nodal explants responded positively. About 80% of the nodal explants responded positively on medium containing sucrose (3%) and NAA + BA (3 µM each in combination) where as many as 12 shoot buds/micro shoots formed without callus formation.

Cultures were also initiated from the foliar explants of *in vitro* source. Swelling of foliar segments was observed within 7 days of culture from the cut ends followed by either

shoot buds formation or callus formation within 3-4 wk. The highest percentage of cultivated leaf segments forming protuberances/shoot buds was 75% and highest mean number of protuberance regenerating buds per segment was 9. Of the different concentration of sucrose tested, better morphogenetic response was achieved on medium fortified with sucrose (3%). For morphogenetic induction, three different PGRs were incorporated at different concentrations. Optimum morphogenetic response was recorded on medium supplemented with BA (6 µM). Under optimum condition ~75% of explants responded positively where as many as 9 shoot buds/micro shoot formed accompanied by callus formation.

The shoot buds developed from the nodal segments and foliar explants were maintained on regeneration medium for culture proliferation and plantlets regeneration. The basal medium was supplemented with different quality and quantity of different organic carbon sources (dextrose, glucose and sucrose at a concentration of 0-4%) and it was found that sucrose enriched medium supported optimum regeneration and culture proliferation. Optimum regeneration of plantlets and culture proliferation was achieved on MS medium fortified with sucrose (3%) and Kn (3 µM) with a mean of 7.3 shoot buds/micro shoots of ~4.5 cm height.

Activated charcoal was also incorporated in the regeneration medium to study its effect on plant regeneration and plant morphology. It was observed that incorporation of AC had very little or no effect on culture proliferation and plant growth. Though AC had very little effect on multiple shoot buds formation but, on AC rich medium plantlets developed better roots. As many as 8 roots per plant formed on medium containing AC (0.1%).

Different substrata like coconut coir, foam and forest leaf litter were also used against agar in the regeneration medium. When compared the regenerative performance on different

substrata including agar, agar gelled medium outperformed the other substrata. On agar gelled medium, there was as many as 7 plants with about 10 roots per plant and plant height was ~4.5 cm. But on foam as substratum, there was only 5 shoots with 3 roots per plant and plant height was ~2.0 cm. On the other hand, cultures maintained on coconut coir supported only 2 plantlets formation while, there was no regeneration on forest leaf litter and cultures degenerated.

The microshoots shoots were treated in two different ways for inducing roots, viz., (i) directly incorporating NAA in the medium and (ii) pulse treating the micro shoots for 3 hr at different concentration of NAA. Pulse treatment with NAA was found to be inferior over incorporation of NAA in the medium. Of the different concentration of NAA used for inducing roots, a concentration of 3 µM supported maximum root growth where as many as 14 roots per plant developed after 4 wk of culture.

The well rooted plants from rooting condition were transferred on ½MS salt solution containing sucrose (2%) and devoid of 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 before transplanting to plastic pots containing a mixture of soil, sand, decayed wood powder at 1:1 ratio with moss topping. The pots were covered with holed transparent polybags and watered at week interval for two months. The plants were fed with $1/10^{th}$ MS salt solution weekly for 3-4 wk and maintained in polyshade with Ca.70% of shading sunlight and finally after two months, the plantlets were left in the normal full day light condition. About 200 plants were tested for survival and about 70% survival was registered after two months of transfer.

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List of Publications

Published as abstracts

1. Arenmongla, T. and Deb, C. R. 2010. *In vitro* culture of immature embryos and mass multiplication of *Malaxis acuminata*. *In*: National Conference on "Orchids: Systematics and Diversity Analysis for Conservation and Sustainable Utilization", GBPIHED, Almora, India. March 19-21. Pp. 88-89.
2. Arenmongla, T. and Deb, C. R. 2011. *In vitro* propagation of *Malaxis acuminata* from different explant sources: A therapeutically important orchid. *In*: National Consultation for "Production and Utilisation of Orchids", NRC for Orchids (ICAR), Pakyong, East Sikkim, India. February 19-21. Pp. 108-109.
3. Arenmongla, T. and Deb, C. R. 2011. *In vitro* plant regeneration and mass multiplication of *Strobilanthes flaccidifolious* Nees. A dye yielding plant of Nagaland. *In*: World Congress for Man and Nature on "Global Climate Change and Biodiversity Conservation", Department of Zoology and Environmental Science, Gurukula Kangri Vishwavidyalaya, Haridwar (U.K), India, November 11-13. Pp. 354

Published/accepted/communicated in peer reviewed journal

1. Deb C. R. and Arenmongla, T. 2011. Morphogenetic induction and organogenic differentiation from foliar explants of *Strobilanthes flaccidifolious* Nees: a natural dye yielding plant. *J. Exp Sci*, 2011, 2(10): 22-28.
2. Arenmongla, T. and Deb, C. R. Asymbiotic immature embryo culture and mass multiplication of *Malaxis acuminata*: A therapeutically important terrestrial orchid. *IndianJ Biotech* (In press).
3. Arenmongla, T. and Deb, C. R. Development of cost effective *in vitro* regeneration protocol of *Malaxis acuminata* D. Don a therapeutically important orchid using pseudobulbs as explants source. *J. Pl Biochem Biotech* (Under Revision).
4. Deb, C. R. and Arenmongla, T. An efficient *in vitro* regeneration system of *Strobilanthes flaccidifolious* Nees from nodal explant: A natural dye yielding plant. *Indian J Exp Biol* (Under Revision).

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