MICROPROPAGATION OF *ILEX KHASIANA* HOOK F. AND *NYMPHAEA TETRAGONA* (AIT.) GEORGE- RARE AND ENDANGERED PLANTS OF MEGHALAYA, INDIA

ABSTRACT

BY

JITEN CHANDRA DANG

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ABSTRACT

India with a large reservoir of diverse plant species is designated as one of the 12 mega diversity hot spots in the world. Home to numerous medicinal and variety of plants, India has both Alpine and Tropical plant species because of its varying climate. From time immemorial man has relied on plants for food and treatment of various kinds of ailments. Population explosion has resulted in the need for land for agriculture, urbanization and other activities. Deforestation of tropical as well as temperate forests on a large scale, shifting cultivation and soil erosion are some of the main factors that have contributed to the depletion of natural biodiversity. There is an increasing pressure on the plants for human survival and economic well being throughout the world. The overexploitation of plants for medicinal and commercial purposes has also degraded the natural genetic resources. Besides, rapid industrialization coupled with increased human activities have resulted much to the shrinking of the natural plant resources of biodiversity.

Due to such rapid destruction of forest resources by mankind, numerous plant species have already become extinct; many more are becoming rare/endangered in their natural habitats. In fact, along with such plants, *Ilex khasiana*, a holly tree and *Nymphaea tetragona* an aquatic lily herb have also been placed in the list of rare and endangered plants. *Ilex khasiana* is used during Christmas decorations. It is one of the few forest trees of Khasi Hills, Meghalaya and has enormous potential for maintaining landscape and healthy environment (or ecosystems) of hilly regions. The main problem with this tree is its ill-rudimentary developed embryo and seeds bearing such embryos can hardly germinate in nature. On the other hand, it cannot be

vegetatively propagated through cuttings. In the past, *I. khasiana* was abundantly present throughout Khasi Hills but at present it is restricted to a few pocket areas of this region. *Nymphaea tetragona,* an aquatic lily has a very low population confined to a single place at Nongkrem Smit pond, Shillong and is on the verge of extinction. This water lily is valuable ornamentally for its showy flowers. The rhizome of the plant is locally used to cure acute diarrhoea and to join bone-fracture.

Keeping in mind the ever increasing threat to plant diversity of the Northeastern region, there is an urgent need to conserve these two important rare and endangered plant species. The proposed study was undertaken with the following objectives:

1. Micropropagation of *I. khasiana* and *N. tetragona* using various explant sources, media, and culture conditions.
2. Developing complete protocol for mass multiplication of these plants.
3. Developing short to medium-term *in vitro* storage methods.

The main methods adopted for the successful completion of the present work are summarized below:

- Explants like apical buds, nodal stem segments, leaf discs, rhizome buds were tested in different seasons for their best performance on the culture media.
- For checking browning of explants from mature trees, various pre-treatments like ascorbic acid 50 mg/l, PVP (0.5%), citric acid (75mg/l), charcoal (1-2%), chilling, distilled water and serial transfer of explants at 2, 4, 8, 12, 24, 48, 96

h intervals were done for maximum survival of the explants on the culture medium.

- Detergents like teepol and soap water were used with different concentrations and varying period of time for thorough cleaning of the explants.
- For the surface-sterilization of explants, the sterilants like $HgCl_2$ solutions, ethanol 70% and Savlon were used with different concentrations and time period.
- Different nutrient media like MS, LS, Nitsch and White were tested for optimising best growth and development of explants in the culture.
- Effects of different cytokinins like BAP, KIN and Zeatin were recorded for the direct multiple shoot bud induction.
- Effect of cytokinin-auxin ratio on the induction of multiple shoots was seen.
- For the initiation of callus from leaf of *I. Khasiana*, effects of different auxins alone and combinations of auxin-cytokinin were observed.
- Induction of caulogenesis from callus was tried in the medium treated with various concentrations of growth hormones.
- For cold storage, *in vitro* obtained shoots of *I. khasiana* and of *N. tetragona* were kept at 4 ^{0}C in dark for a period of upto 8 months and the regeneration capacity and duration of survival were tested at monthly intervals.
- Various pre-treatments like reduced supply of nutrients, low temperature treatment, addition of ABA, addition of mannitol or sugar for low osmoticum were tested for the optimal cold storage conditions.

- Easy and cost effective methods for the successful hardening and acclimatization of *in vitro* obtained plantlets were investigated.

Main Outcomes of the Present Study:

The important outcomes of the present research work are summarized below:

- The suitable season for explant collection for direct multiplication of shoot regeneration was found to be April to July in both the cases.
- For surface sterilization, initial treatment of the explants with 0.5% teepol detergent for 30 minutes followed by Na OCl (10%) or $HgCl_2$ (0.01-0.1%) solution for 10-15 minutes, 70% ethanol treatment for 10- 30 seconds and inoculation to the medium showed maximum survival of the explants in the culture medium.
- In case of *I. khasiana*, the browning of the explants collected from mature trees, could be checked, by transferring explants serially on to the fresh medium with 0.5% PVP at 2,4,8,12,24,48,96 h intervals.
- To reduce/overcome the problem of browning and death of the explants from mature trees of *I. Khasiana,* the *in vitro* raised seedlings from seeds were used. The percentage of seed germination was recorded to be only 5-10 % after 2 ½ months.
- The most suitable explants for direct shoot multiplication of *I. khasiana* was the nodal explant from the young seedlings. The average number of shoots obtained was 10-11 shoots per explant in MS medium containing cytokinins BAP (2.0 mg/l) + KIN (1.0 mg/l).

- In case of *N. tetragona*, cultured rhizome buds proliferated multiple shoots in MS medium incorporated with BAP (2.5 mg/l) + IAA (0.5 mg/l). Within 4-6 weeks an average number of 3-4 shoots were produced.
- The premature embryo culture of *N. tetragona* was successfully carried out and the number of multiple shoots was 12 per embryo in MS medium incorporated with BAP (2.0 mg/l) + NAA (0.5 mg/l).
- For *in vitro* rooting, MS half- strength medium supplemented with IBA (2.0 mg/l) was found to be optimal in case of *I. khasiana* and hormone-free MS medium in *N. tetragona.*
- Among different explants used, leaf discs of *I. khasiana* responded very well and within 4 weeks callus induction could be obtained.
- MS medium supplemented with 2, 4- D (2.0 mg/l) + KIN (0.5 mg/l) was the optimum for the induction of callus.
- Shoots could be induced from the callus of *I. khasiana* in ½ MS medium containing a combination of 1.5 mg/l BAP and 0.5 mg/l IBA.
- For elongation and development of plantlets from callus, gradual removal of 2,4-D from the medium was necessary.
- The cold storage period of *in vitro* shoots of *I. khasiana* was upto 6 months whereas in case embryo of *N. tetragona* the storage period was 5 months.
- Among the different effects of minimal growth conditions on the survival and multiplication of shoots after, the treatments of reduced ½ strength MS and 5.0 mg/l ABA in MS medium showed the best survival and multiplication rate.

- Plantlets produced by direct multiple shoot induction showed 60 % survival after hardening in *I. Khasiana.*
- Plantlets produced indirectly through callus showed 40 % survival after hardening.
- Plantlets obtained directly from rhizome and embryo explants of *N. tetragona* showed 50 % and 60 % survival respectively after hardening.

Conclusion and limitation

Plant tissue culture technique, an integral part of biotechnology deals with the mass propagation and conservation of elite clones and varieties. Recent advances on plant tissue culture coupled with genetic engineering made possible to obtain GMO's (Genetically Modified organisms) to increase the overall productivity, with quality and disease/drought resistance of desired traits. Callus mediated plantlets have potential for somaclonal variations for commercially improved plants. The recent trend of emerging plant tissue culture along with molecular biology can provide integrated and valuable information particularly in understanding the genetic control of each development of callus, caulogenesis and plantlet development. The protocols developed for mass multiplication could easily be adopted for the large scale propagation of important plants with a view to sustainable conservation.

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SHILLONG

OCTOBER 2005

DECLARATION

I do hereby declare that the thesis entitled "Micropropagation of *Ilex khasiana* Hook F. and *Nymphaea tetragona* (Ait.) George- Rare and Endangered Plants of Meghalaya, India" is a record of original and independent research work carried out by me in the Department of Botany, North-Eastern Hill University, Shillong under the supervision of Prof. Pramod Tandon. The work done is original and no part of the thesis has been submitted for any other degree or diploma of any university.

Jiten Chandra Dang

North-Eastern Hill University

Shillong

Date 26/10/05

Head 26/10/05

Head

Department of Botany

School of Life Sciences

N.E.H.U., Shillong-22

Pramod Tandon

Supervisor 24/10/05

Telefax Work: +91 364 255 0300 ☎Work: +91 364 272 2214/ 272 2244/ 255 0150; Home: +91 364 255 0100
E-mail: tandon1@sancharnet.in / profptandon@yahoo.com

North-Eastern Hill University

Professor Pramod Tandon

Plant Biotechnology Laboratory
Department of Botany, School of Life Sciences
Shillong 793 022, India

CERTIFICATE

I certify that the thesis entitled "***Micropropagation of Ilex khasiana Hook F. and Nymphaea tetragona (Ait.) George - Rare and Endangered Plants of Meghalaya, India***," submitted by Mr. Jiten Chandra Dang for the degree of Doctor of Philosophy in Botany Department of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. Degree. This work has not been submitted for any degree of any other University.

Pramod Tandon

(Pramod Tandon) 24/10/05

Supervisor

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J. C. Dang

CONTENTS

ABBREVIATIONS USED

AC	-	Activated Charcoal
BAP	-	6-benzylaminopurine
B_5	-	Gamborg's medium
GA_3	-	Gibbrellic Acid
IAA	-	Indole -3- acetic acid
IBA	-	Indole -3-butyric acid
KIN	-	Kinetin
LS	-	Linsmaier and Skoog medium
mg	-	Milligram
MS	-	Murashige and Skoog medium
2,4-D	-	2,4-dichlorophenoxyacetic acid
NAA	-	$\propto$-Naphthalene acetic acid
PVP	-	Polyvinyl pyrrolidone
UV	-	Ultraviolet

CHAPTER I: INTRODUCTION

India is abundantly endowed with 45,000 species of plants which accounts for 8% of global genetic resources. It, therefore, comes as no surprise that this country has been designated as one of the 12 megadiversity spots in the world. Besides being a home to numerous medicinal and other variety of plants, India has both alpine and tropical species because of its varying climatic conditions (Banerjee, 1998). The Northeastern region of India alone harbours about 45% of the country's flora (Nayar, 1997; Rao, 1997).

Forests, natural reserves, uncultivated lands and homeyards are the main habitats where genetic resources of rare/endangered species, orchids, medicinal and aromatic plants are found. Genetic variability which exists in these habitats, is a necessity for improvement programmes and conservation of germplasm.

Population explosion has resulted in the need for land for agriculture, urbanization and other activities. Consequently, tropical forests are disappearing and undergoing extreme modifications at an alarming rate. The disappearance of natural resources and shrinking of habitats, together with the spread of advanced technology in agriculture, particularly selection and breeding of economically important plant species led to the loss of variability in crops , a process known as "genetic erosion". The over exploitation of plants for medicinal and commercial purposes has depleted the genetic resources which has resulted in the degradation of biodiversity. Therefore, due to the rapid industrialization and increased human activities, the natural plant resources of biodiversity have been shrinking day by day (Akrele, 1991).

There is increasing pressure on the plants for human survival and economic well being throughout the world. At the global level 1 in 8 of living plants is endangered (IUCN, 1998). India being no exception to this where 15-20% plant species are considered to be threatened.

Certain medicinally and ecologically important plants such as *Cinchona ledgeriana*, *Cinnamomum zeylanicum*, *Rauvolfia serpentina*, *Taxus wallichiana* are reported to be endangered (Singh and Pruthi, 1998).

Concerted efforts are required to combat this menace of unabated exploitation together with evolving conservation strategies for natural habitats as well as artificial methods of mass multiplication of rare/endangered plant species for their rehabilitation. If remedial measures are not devised there will be a large scale genetic wipe-out of plant resources from the region.

Germplasm conservation in natural habitat is more preferable, but it is becoming more difficult and in certain cases impossible . For such situations *ex situ* conservation becomes inevitable for which modern plant science technologies have to play a major role. The main principle of genetic conservation is to store plant germplasm for future use. It is generally stored in the gene banks, in field conservation or in their natural habitats. Genetic conservation is one of the most important and urgent tasks faced by to-day's plant scientists. Importance of genetic conservation principle is more applicable in the tropics, where variety of genetic diversity exists. The conservation of endangered plant species so as to prevent from further extinction is one of the top priorities of to-day's plant scientists throughout the world.

Conservation of plant genetic resources could be accomplished both by *in situ* and *ex situ* methods (Tandon, 2004). *In situ* conservation involves protection of genetic resources in the natural environment through the protection of the environment itself. *Ex situ conservation* programmes aim in acclimatization, rehabilitation, multiplication and judicious exploitation. The role of biotechnology is complementary to conventional conservation methods and can directly assist plant conservation programs through molecular marker technology, molecular diagnostics, *in vitro* technologies and cryopreservation (Tandon, 2000; Tandon and Kumaria, 1998). Conservation of germplasm is appropriate to those plants which do not produce any seeds or produce recalcitrant seeds , or which are large trees difficult for field conservation. The technology of preservation of plant cells and organs and its potential application for long term storage of plant germplasm has been proposed (Henshaw, 1979; Withers and Bajaj 1979; Withers ,1980).

One of the most useful exploitations of plant tissue culture technique has been the clonal propagation to reproduce plants on mass scale of desired genotypes (Murashige, 1978; Vasil and Vasil 1980; Conger, 1981; Tomes *et al.*, 1982). This is more relevant to extremely reduced populations or rare

/endangered species for which conventional methods of sexual or vegetative propagation become a limiting factor. It offers many unique advantages over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease - free plants, non-seasonal production, secondary metabolite production and germplasm conservation.

The concept of totipotency of a plant cell was practically demonstrated by Vasil and Hilderbrandt (1965) where a single isolated cell in the medium could divide and ultimately grow into a whole plant. Guha and Maheshwari (1964) successfully produced haploid plants in *Datura innoxia* for the first time. Plant regeneration from isolated protoplasts was accomplished by Takebe *et al.* (1971) in *Nicotiana tabaccum* and the first somatic hybrid plant was obtained by Carlson *et al.* (1972).

Preservation of germplasm is a means to assure availability of genetic materials as the need arises. Since most seeds and vegetative organs have limited storage life, research in germplasm preservation has concentrated on the development of procedure to extend usable life spans. Cold storage of germplasm involves the maintenance of under minimal growth conditions. The reduction of salts in the medium and treatment of low temperature (1-9°C) are used to slow down the rate of metabolic activities. Cold storage of cultures under aseptic conditions at low temperature shows minimal rates of growth. Grape plants could be stored for over 15 years at 9°C by early transfer to freeze medium (Manzhel *et al.*, 1983). This approach to plant storage is very simple and gives high rate of survival. *In vitro* cold storage through minimal growth techniques have been applied to meristem and shoot cultures of some forest species with varying successful storing periods for e.g., 10 months for *Euclayptus citriodora* (Mascarenhas and Agarwal, 1991), 60 months for *Populus* spp. (Hausman *et al.*, 1994). Watt *et al.* (1996, 2000) mentioned that the inevitable presence of endogenous bacterial and fungal contaminants encountered in *Eucalyptus* cultures must be taken into consideration when managing germplasm storage.

In order to achieve the goal of germplasm conservation, cell, tissue and organ culture must be an integral part of the whole operation. The credit of modern technology of plant cell and tissue culture goes to Haberlandt (1902), a German Botanist, who originally carried out in pioneering experiments on growing and maintaining isolated plant cell in a viable liquid plate of simple defined nutrient medium. The advances in plant tissue culture have made significant contributions in cell biology, physiology, biochemistry and molecular biology (Bhojwani and Razdan, 1996). The more practical applications are the techniques of clonal multiplication, development of somaclonal variants, production of secondary metabolites and improvement of plants through somatic hybridization and genetic manipulation. The tools of biotechnology are being increasingly applied now for conservation of plants (Prance, 1997; Feijoo and Iglesias, 1998; Lynch, 1999).

Plant cell and tissue culture has become a powerful tool for studying basic and applied problem of plant sciences having a wide range of commercial application in horticulture, agriculture and forestry (Bonga, 1982). The advantage of micropropagation of plants is that multiplication cycle is very short, and can be continued throughout the year irrespective of seasons. Although a great deal of research work on clonal propagation of desirable plants through tissue culture is now in progress, the tissue culture of trees has lagged behind. Morovere, most of the trees are difficult to propagate vegetatively in comparison to herbaceous plants. There are a few successful reports covering whole range of tree propagation through tissue culture (Mohan Ram *et al.*, 1981; George, 1996). However, many hurdles have been encountered in the regeneration of tissues derived from woody species (Sita *et al.*, 1979; Zimmerman, 1983; Roy and Datta, 1985; Kapoor and Gupta, 1986; Bajaj, 1986; Bonga and Durzan, 1987; Bejoy and Hariharan, 1993).

Basic nutritional composition of different tissue culture media were adopted by many workers as Gautheret (1955), Nitsch (1951) Murashige and Skoog (1962), White (1963), Gamborg et al. (1968), Schenk and Hildebrandt (1972) and WPM (Lloyd and McCown, 1980) with respect to kinds and concentrations for particular plant species ranging from mosses to angiosperms. Nutritional requirements for optimal growth of tissue *in vitro* may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962).

Since the tree species are not amenable to vegetative propagation by rooting of excised branches or by grafting tissue culture method is attracting considerable attention for obtaining pure, elite populations under *in vitro* conditions (Sita *et al.*, 1982). The special growth characteristics of trees pose difficult problems for tissue culturists, as they are slow growing coupled with dormancy and calli in particular when excised from mature trees are rarely responsive to conditions designed to stimulate plant regeneration (Anand and Bir, 1984).

The mass propagation of plantlets through tissue culture, utilizing bud or meristem culture will ensure the progeny with genetically identical to the parent plant. Shoot tip or meristem culture can eliminate diseases because such explants do not posses xylem or phloem elements. Conservation of plant materials through cold storage technique has gained as a novel delivery of propagules and extend this technique for the long-term storage of genetically valuable plant materials of germplasm.

Considering all these into account, the present research work deals with developing methodology for mass propagation of *Ilex khasiana* Hook. F. and *Nymphaea tetragona* (Ait.) George, - two rare and endangered plants of Northeast India and their preservation as a strategy to conserve the vanishing germplasm.

Ilex khasiana (Aquifoliacaea), a small forest tree, is restricted to certain pocket areas of Khasi Hills of Meghalaya. The tree has dark green leaves, small showy flowers and red berries (Photoplate 1.1). The plant has ritual importance as it is used to decorate churches and houses during Christmas. This is one of a few hilly forest trees of Khasi Hills which has biomass potential and land scaping purposes to maintain healthy ecosystem. The ruthless exploitation of forest trees of this region has made this tree species rare in its natural habitats. It has resulted in its distribution restricted in limited pocket areas of Meghalaya in Northeast India.

Nymphaea tetragona (family Nymphaceae), an endangered aquatic herb is found only at Nongkrem Smit pond in Khasi Hills (Photoplate 1.2 and 1.3). A very small herb has highly restricted distribution with only a few representatives found in fragile ecosystems vulnerable to anthropogenic activities. The showy white to pinkish flowers have ornamental aquario value. The rhizome of *N. tetragona* is locally used to cure acute diarrhoeae and dysentery. It is also used to join bone-fractures. The plants fail to regenerate in nature because of incomplete development of embryos and they are easily susceptible to pests and diseases too. These lacunae of the plant lead to its poor regeneration. As a result the population of this plant species is on the verge of extinction.

Water plants play an important key role in aquatic ecosystem and serve a variety of purposes such as food, fibre, paper-pulp, green manure, mineral recycling and control of pollution (Gujral *et al.*, 1986). The established benefits of plant tissue culture in micropropagation, production of haploids, disease detection and eradication, elimination of breeding barriers, biosynthesis of secondary metabolites, generation of variability and in conservation of germplasm can be exploited in aquatic plants (Mohan Ram, 1991; Mohan Ram and Agrawal, 1993).

Keeping in mind the ever increasing threat to plant diversity of the North-Eastern region, there is an urgent need to conserve it for posterity. The proposed study was aimed at micropropagation of . *I. khasiana* and *N. tetragona* using various explant sources, media and culture conditions, developing complete protocol for mass multiplication of these plants and developing short to medium-term *in vitro* storage methods.

Photoplate: 1.1

10-12 yrs old *Ilex khasiana*


Photo plate: 1.2

Nymphaea tetragona plants growing in June at Nongkrem Smit pond, Shillong

Photoplate 1.3

Nymphaea tetragona plants in July at Nongkrem Smit pond

CHAPTER II: REVIEW OF LITERATURE

The tools of biotechnology are being increasingly applied now for conservation of plants (Prance, 1997; Feijoo and Iglesias, 1998; Lynch, 1999). A large number of plants have been micropropagated using different explant sources, media and culture conditions. Compared to the traditional propagation, micropropagation is advantageous. It leads to simultaneous accomplishment of rapid large-scale propagation of new genotypes, the use of small amount of original germplasm and generation of pathogen-free explants (Withers and Engelmann, 1998). A number of endangered plant species have been re-established in their natural habitat using *in vitro* propagation methods (Tandon *et al.*, 1990; Tandon and Rathore, 1992, 1994; Seeni and Sabu, 1997; Ramsay and Stewart, 1998). However it is difficult to generalize the cultural practices used. The reestablishment of a large number of micropropagated plantlets into their natural habitat is of prime concern, as some endangered plants may not reproduce well by conventional methods. Methods such as micropropagation and somatic cell culture can be useful for propagation, genetic improvement and germplasm conservation of plants of the region. Pénce (1999) has extensively reviewed the use of *in vitro* propagation methods for conservation of endangered plants. The micropropagation of endangered species is at times hampered due to limited availability of the material for raising cultures. In such cases, micropropagation protocols established for the related non-endangered plants are of great help in germplasm conservation. However, a suitable technology needs to be developed for each of the endangered species. To meet the predicted requirements of timber and to conserve the forests the potential of micropropagation in forestry improvement has been reviewed (Ahuja, 1993).

In most of these studies, either apical or axillary buds , nodal segments, rhizome tips, leaves, inflorescence, petiole, embryo, seed, were taken from mature plants or shoot tips hypocotyls, cotyledon or cotyledonary nodes from aseptically germinated seedlings or embryo axis from zygotic embryos etc., were used as the explant source.

Previous reports on micro propagation of rare/endangered plants include - shoot tip culture in *Picrorhiza kurroa* (Lal *et al.*, 1988), micropropagation of *Coleus forkohlii* (Sharma *et al.,* 1991); *Leontochir ovallei* (Lu *et al., 1995); Gymnema elegans (Komalavalli and Rao, 1997); axillary* bud culture in *Rotula aquatica* (Martin, 2003), leaf culture in *Dionaea muscipula* , Jang *et al.,* 2003), leaf and petiole culture in *Thapsia garganica* (Makunga *et al.*, 2003), zygotic embryo culture in *Podophyllum hexandrum* (Arumugam and Bhojwani, 1990 and in *Hyophorbe lagenicaulis* (Sarasan *et al.*, 2002), epicotyl culture in *Saussurea obvlilata* (Joshi and Dhar, 2003), nodal segments culture in *Symonanthus bancroftii* (Panaia *et al.*, 2000).

Micropropagation of tree species offers a fast means of producing stock for afforestation and conservation of elite and rare germplasm. The rate of depletion of forest resources is much higher than the rate of forest regeneration, which results in ecological imbalance. Therefore the development and standardization of protocols for *in vitro* multiplication and transfer of plantlets from laboratory to field for afforestation, wasteland development, agroforestry and social forestry have to be developed. Some micropropagation of endangered tree species were carried out using tissue culture technique, e.g., *Sterculia urens* (Purohit and Dave, 1996; *Saraca asoka* (Harikrishnan *et al.*, 1998).

The *in vitro* seed germination has been extensively used for multiplication of a large number of orchid species (Arditti and Ernst, 1984; Sharma and Tandon 1987; Yam *et al.*, 1989; Kumaria and Tandon, 1991; Kondo *et al.*, 1997; Gangaprasad *et al.*, 1999) and could provide rapid means of multiplying rare and endangered orchids. In certain cases, explants from the *in vitro* raised seedlings are used to initiate cultures for micropropagation (Sharma *et al.*, 1992; Corrie and Tandon. 1993; Hardy *et al.*, 1995). Seeds are preferred for multiplication of rare and endangered species, as this would ensure genetic diversity. *In vitro* techniques can also be used for the rapid and clonal propagation of various medicinal plants of the region and thus pave the way for conservation and economic utilization of such high value plants. Sharma *et al.* (1995) have reported *in* vitro mass multiplication of a number of medicinal plant species such as *Gentiana kuroo, Picrorhiza kuroa, Coleus forskohlii, Saussurea lappa* and *Tylophora indica* , which are on the verge of extinction.

Short- to medium-term storage of plant germplasm has been accomplished by reducing the temperature at which the cultures are grown. However, the responses of different cultures vary. For instance, it has been reported that the cold tolerant species such as strawberry and *Prunus* species can be stored at 0°C to 4°C (Wilkins *et al.*, 1988; Reed 1992) whereas *Musa* plantlets cannot be stored below 15 °C such as mannitol, reduction of growth regulators, strength of the nutrients and the use of growth retardants have resulted in the slow growth of cultures (Staritsky and Zandvoort, 1985; Jarret and Gawel, 1991; Malaurie *et al.,* 1993). The short-term conservation of germplasm by the use of mineral oil overlay of cultures has been achieved (Constable and Shyluk, 1994). The mineral oil overlay lowers the oxygen levels. The slow-growth storage of cultures has certain drawbacks, for instance the management of large *in vitro* collections and the possible development of somaclonal variations in cultures.

In vitro collections, which are maintained under short-term storage, require manpower immensely. The *in vitro* plants can be maintained for long periods or 'long-term storage' by reducing the growth rate which can be achieved by temperature reduction, light intensity reduction, growth regulators use, limitation of minerals supply, addition of osmotic stress agents, or by the combination of any of these methods.

Since the majority of trees are not amenable to vegetative propagation, tissue culture application is attracting considerable attention for obtaining pure and elite populations under *in vitro* conditions (Sita *et al.,* 1982). The main hurdles in propagation of trees are non-availability and recalcitrant nature of seeds (Luna, 1989). Trees pose difficult problems for tissue culturists as they are slow growers and the calli obtained from explants obtained from trees are rarely with regenerative potential (Anand and Bir, 1984). However, some success in tropical tree fruits, leguminous tree species and forest trees have been reported (Mascarenhas and Muralidharan, 1989; Mittal *et al.*, 1989; Wu *et al.*, 1990; Jaiswal and Gulati, 1991; Bhatt *et al.*, 1992; Jana *et al.*, 1992; Gavish *et al.*, 1992; Mathur and Mukuntha Kumar, 1992; Swamy *et al.*, 1992; Brand , 1993; March *et al.*, 1993; Kim *et al.*, 1994; Das *et al.*, 1997; and; Sharma and Chandra, 1998; Xie and Hong, 2001 and Mathur and Hernandez *et al.*, 2003).

Successful induction and development of multiple shoots and regeneration of plantlets from axillary bud/shoot tip/ nodal segments from mature trees have been achieved in many cases. Such as axillary bud culture in *Eucalyptus* (Das and Mitra 1990), *Aegle marmelos* (Ajithkumar and Seeni, 1998), *Mitragyna* (Roy *et al.*, 1988), nodal shoot segment culture in *Tecomella* (Rathore *et al.*, 1991), shoot tip and node culture in C*calophyllum* (Nair and Seeni, 2003), *Taxus* (Chang *et al.*, 2001, *Syzygium* (Roy *et al.*, 1996), shoot tip culture in *Mangifera* (Thomas and Ravindra, 1997). Multiple shoots from seedling explants of tree species have also been developed in *Acacia* (Mittal *et al.*, 1989), *Murraya* (Bhuyan *et al.*, 1997), *Syzygium* (Jain and Babbar, 2000) *Acer* (Durkovic, 2003).

Rao (1965) reported for the first time embryo development from the cultures of *Santalum album* which failed to grow into complete plants. Radojevic (1979) studied ultra-structural aspects of somatic embryogenesis in *Paulownia tomentosa* and distinguished two different patterns of origin of somatic embryos from *in vitro* grown explants : viz., (i) direct production of somatic embryos (ii) indirect production of somatic embryos from callus.

Although somatic embryogenesis have been reported in many plants, difficulties were encountered in the regeneration of tissues derived from woody species (Bajaj, 1986; Bonga and Durzan, 1987) Plantlet regeneration from somatic embryogenesis have been reported from a few limited adult trees on *Santalum* and *Dalbergia* (Sital *et al.*, 1979; Rai *et al.*, 1988), *Pyrus* (Chevreu *et al.*, 1989) *Malus* and *Cerasus* (Tanto *et al.*, 2001) *Quercus* (Garcia *et al.*, 2001 and Hernandez, 2001). Somatic embryogenesis from seedling explants have also been reported in several tree species *Albizzia* (Datta, 1987), *Eucalyptus* (Das and Mitra, 1990), Hariharan, 1991), *Citrus* (Gill *et al.*, 1991), rubber (Etienen *et al.*, 1993), *Acacia* (Skolmen, 1986; Xie and Hong, 2001, and *Pinus* (Deb and Tandon, 2002).

In angiosperms, the major obstacle faced in many cases in the low rate of germination and maturation of somatic embryos into complete plantlets. However, in some cases, more than 10 % of the somatic embryos grow into whole plants eg., *Coffea arabica* , *Santalum album, Liriodendron tulipifera* and *Corylus avellana* (Sita *et al.*, 1979; Merkle and Sommer, 1986; Perez *et al.* 1983).

The genus *Ilex* has ill rudimentary embryos that remain immature at heart-shaped stage for a long time even after the fruits reach maturity (Martin, 1946; Anonymous, 1948; Kosar, 1959; Barrett, 1962). A minimum of one year under the proper germination conditions is required by seeds of *I. aquifolium* and *I. cornuta* from mature berries to complete their embryogenic development before germination (Kosar, 1959). Barrett (1962) referred to this period of embryonic development (from heart shaped stage to mature embryos) of *I.*

opaca as "late embryony". Hu (1968) reported *in vitro* culture of rudimentary embryos of eleven *Ilex* species to by-pass this period of seed dormancy.

Hu and Sussex (1972) isolated heart shaped embryos from the seeds of *I. aquifolium* (English holly) and cultured in Linsmaier and Skoog (LS) medium wherein embryos completed development and germinated in 6-8 weeks. Further, Hu *et al.* (1979) studied the acquiescent state and structural changes during culture. A micropropagation system was developed for *I. vomitoria* (Yaupon) hollies from shoot tips (Avirineni and Ford, 1990). Sansberro *et al.*, (1999) studied *in vitro* plant regeneration of *I. paraguariensis* using nodal segment, shoot tips and apical meristematic dome.

About 470 species of flowering plants constitute the aquatic flora of the Indian subcontinent accounting for nearly half of the world's known aquatic augiosperms (Biswas and Cadler, 1937; Subramanyam, 1962; Deb 1976; Gopal, 1990; Rao, 1997). The vast potential of water plants is only marginally utilized and scientific approach has not been developed for deriving greater economic and ecological benefits (Mohan Ram, 1991).

Cook (1990) has attempted to define aquatic plants as all seed bearing plants whose photosynthetically active parts are permanently or at least, for several months each year are submerged in water or float on the surface of water.

The systematic relationship of aquatic flowering plants are varied, yet they share many similar characteristics necessitated by common habitat (Sculthorpe, 1967). These include presence of air cavities and development of special structures for buoyancy, reduction in mechanical and vascular tissues adaptation in the mode of pollination and fruit/seed dispersal. However, the degree of adaptation may differ based on their surroundings whether the plants are amphibious , attached, free floating, emergent or submerged.

Although commendable progress made with land plants, utilization of results of tissue culture has not been impressive and published accounts on tissue culture of water plants are limited (Mohan Ram and Kakkar, 1983; Kane, 1989; Mohan Ram, 1991).

Besides regulating key ecological processes, aquatic plants serve a variety of purposes such as sources of good, fiber, paper, pulp, green manure and for mineral recycling and control of pollution (Boyd, 1972; Gujral *et al.*, 1986).

Due to the increase of human population and explosion, tremendous pressure on land is mounting and the alternative or compulsive demand for the study and utilization of marine and fresh water ecosystems is not difficult to realise. Plant tissue culture could be useful for this purpose.

Besides food, fiber etc., aquatic plants are used as pollution indicators and absorb pollutants like sulphur dioxide, ozone, pesticides and chemicals that are polluted in water due to human interventions. They are also useful in accumulating heavy metals in large amounts. Arsenic is absorbed by *Ceratophylum* up to 26 ppm with no adverse effects. *Spirodela* (a duckweed) is reported to have yielded zinc at a concentration factor of about 25,000 (Hutchinson, 1975). Other aquatic plants such as *Lemna*, *Myriophyllum*, *Scirpus* and *Pistia* have also been used in the treatment of industrial waste waters and nutrient agricultural drainage.

The utilization of aquatic weeds for this purpose will require well designed programmes to pick up such native species which can selectively take up heavy metals and other carcinogenic substances. For accurate, quick and dependable results, the *in vitro* tissue culture technique should prove valuable. Moreover, the merit of the *in vitro* system lies in the fact that efficiency of a very large number of herbicidal compounds could be tested on many aquatic weed species, cultured *in vitro,* even in season when they do not occur in nature.

In general hydrophytes are poor in secondary metabolites. Very few medicinal principles have been isolated and identified from them (Sculthorpe, 1967). Yet traditional used of aquatic plants have been reported from India (*Acorus calamus*, *Hygrophila spinosa*, *Bacopa monieri*, etc.). The extraction of growth hormones from the roots of *Eicchornia* was also reported (Bhanja *et al.*, 1967).

Growing whole plants rather than only their parts under precisely controlled and aseptic condition provides extensive opportunities to understand problems of organization, integration and interaction. Aquatic plants are specially favourable for this purpose because of the ease which they can be propagated, grown and multiplied in liquid culture (Mohan Ram, 1991).

The convenience with which nutrients can be supplied makes the axenic culture of whole hydrophytes ideally suited for understanding chemical ecology, to do critical analytical work dealing with metabolism and to visualize fine structure.

Thus, successful cultures have been raised in *Limnophilla* (Rao and Mohan Ram, 1981) , *Neptunia* (Kakkar and Mohan Ram, 1986), *Nelumbo* (Kane *et al.*, 1988b; 1990 b) and *Trapa* (Agrawal, 1993).

Early attempts to germinate the seed of podostemads and to raise plants were unsuccessful. When the seeds of some members of the podostemaceae such as *Griffithella*, *Indotristicha*, *Polypleurum*, *Hydrobryopsis*, *Dalzellia* were transferred to a nutrient medium aseptically, they failed to germinate. However, a novel technique of planting seeds on a cube of thermocole (polystyrene foam) and floating the cubes on the sterile medium , led to high rates of germination (Vidyashankari and Mohan Ram, 1987).

Now simple, rapid reproducible protocols have been established to complete the from seed germination to flowering cycle in the members of podostemaceac family such as *Griffithella* (Vidyashankari and Mohan Ram 1987), *Indotristicha* (Vidyashankari, 1988), *Polypleurum* (Sehgal *et al.*, 1993) where the family Podostemaceae represent embryologically unique pseudo-embryosacs, presence of paired pollen, absence of double fertilization and lack of endosperm.

Commercial micropropagation of aquatic plants has been limited to two end uses : aquaria and horticulture . These include *Cryptocoryne lucens*, *Echinodorus*, *Nelumbo lutea* (lotus), *Nymphaea* species (water lilies), *Myriophyllum* and *Potenderia* (Kane *et al.*, 1988; 1990a, b; 1991 a, b; Jenks et al., 1990; Lakshamanan, 1994). Micropropagation of direct shoot organogenesis has been reported in several aquatic plants such as rhizome tip culture in *Dionaea* (Parliman et al., 1982). Direct shoot organogenesis from cortical tissue of cultured shoot tips has been observed in *Limnophila indica* (Rao and Mohan Ram, 1981). Adventitious shoots on leaf explants of *Myriophyllum heterophyllum* and internodal stem explants of *M. aquaticum* typically arise from the epidermal cell layer (Kane and Albert, 1989; Kane et al., 1991).

Micropropagation on indirect shoot organogenesis from callus tissue have been reported in a few limited aquatic plant species. Sporadic information exists, describing callus induction and plantlet regeneration, such as from leaf explants of *Neptunia oleracea* and *M. aquaticum* (Kakkar and Mohan Ram, 1986; Kane *et al.*, 1991) and frond culture of *Lemna* (Chang and Chiu 1978; Chang and Hsing, 1978; Slovin and Cohen, 1985 and Frick, 1991). Although information is limited, both direct and indirect shoot organogenesis has been demonstrated in several aquatic plant species (Rao and Mohan Ram, 1981; Kakkar and Mohan Ram, 1986; Kane and Albert, 1989; Kane *et al.*, 1991, 1993) Jenks *et al.*, 2000).

Some studies have been made on regeneration of aquatic plants using *in vitro* methods (Kane and Sheehan, 1988; Kane *et al.*, 1988; Kane *et al.*, 1990 a, b; Kane *et al.*, 1991). Jenks *et al.* (1990) studied *in vitro* establishment and epiphyllous plantlet regeneration of of *Nymphaea* "Daubeniana' using Murashige and Skoog (MS) medium. Rhizome tips, young leaves and developing flower buds were used for *in vitro* plant regeneration and multiplication of winter-hardy water lily *Nymphaea 'James Brydon'* a hybrid of *Nymphaea alba* var. *rubra Nymphaea candid* and *Nymphaea laydekeri* (Lakshmanan, 1994).

Table 2.1: Some of the important micropropagated trees

Species	Explants	Authors
Acacia arabica	Somatic embryogenesis from immature zygotic embryos	Nanda and Rout, 2003
Acacia catechu	Plants obtained from nodal segments	Kaur *et al.*, 1998
Acacia catechu	Somatic embryogenesis from immature cotyledons	Rout *et al.*, 1995
Acacia mangium	Multiple shoots from nodal buds	Bhaskar and Subhash, 1996
A. mangium	Somatic embryogenesis from zygotic embryos	Xie and Hong, 2001
Acacia nilotica	Somatic embryogenesis from endosperms	Garg *et al.*, 1996
Acer caudatifolium	Plantlet regeneration from axillary bud and petiole segments	Durkovic, 2003
Aegle marmelos	Plantlets from nucellus callus	Hossain *et al.*, 1994
Anacrdium occidentale	Plantlets from cotyledonary nodes	Das *et al.*, 1996
Annona muricata	Shoot regeneration form internodal explants	Lemos and Baker, 1998.
Annona muricata	Plantlets from hypocotyls and cotyledon segments	Bejoy and Hariharan , 1991
Azadirchta indica	Plantlets from shoot tips	Joshi and Thengane, 1996
A. indica	Plantlets from zygotic embryos	Chaturvedi *et al.*, 2004
A. indica	Somatic embryogenesis from hypocotyls and cotyledon segments	Su *et al.*, 1997
Bauhinia purpurea	Shoots from stem segments	Kumar 1992
Bauhinia vahlii	Shoots from cotyledonary nodes	Upreti and Dhar, 1996.

Bauhinia variegata	Shoots from nodal segments	Mathur and Mukunthakumar, 1992
Camellia limon	Somatic embryogenesis from styles	Carimi *et al.*, 1994
Camellia sinensis	Multiple shoots from nodal segments	Jha and Sen, 1992
Camellia sinensis	Somatic embryogenesis from immature leaf	Kato, 1996
Camellia sinensis.	Multiple shoots from cotyledon and nodal segments	Bag *et al.*, 1997
Cinnamomum camphora	Multiple shoots from shoot tips and nodal segments	Babu *et al.*, 2003
Citrus aurantifolia	Shoots from nodal segments	Bhat *et al.*, 1992
Citrus reticulata	Somatic embryogenesis from immature embryos and young seedlings (leaf, root, epicotyl, cotyledon etc.)	Gill *et al.*, 1991
Commiphora wightii	Shoots from axillary buds	Barve and Mehta, 1993
Dalbergia latifolia	Shoot buds from mature tree callus	Rao, 1986
Dalbergia latifolia	Plantlets from shoot callus of mature trees	Rai and Chandra, 1988
Dahbergia sisso	Multiple shoots from nodal	Gulati and Jaiswal, 1996
Dahbergia sissoo	Somatic embryogenesis from immature zygotic embryos	Das *et al.*,1997
Eucalyptus tereticornis	Shoots from axillary buds	Das and Mitra, 1990
Ilex aquifolium	Plantlet regeneration from apical and axillary buds	Morte *et al.*, 1992
Ilex opaca	Shoot regeneration from juvenile leaves	Mattis *et al.*, 1995

Ilex paraguariensis	Multiple shoots from nodal segments	Sansberro *et al.*, 1999
Ilex vomitoria	Plantlet regeneration from shoot tips	Avirineni and Ford, 1990
Juglans regia	Somatic embryogenesis from immature fruits	Preece *et al.*, 1995
Malus domestica	Somatic embryogenesis from cotyledon of immature zygotic embryos	Daigny *et al.*, 1996
Mangifera indica	Somatic embryos from nucellus	Jana *et al.*, 1993
Mangifera indica	Shoots from leaf segments	Raghuvanshi and Srivastava, 1995.
Mangifera mdica	Somatic embryogenesis from ovule	Pliego-Alfaro *et al.*, 1996.
Mangifera laevigata	Multiple shoots from nodal segments	Islam *et al.*, 1993
Mimosa tenuiflora	Multiple shoots from auxillary buds	Villareal and Rojas, 1996
Murraya koeinigii	Shoot regeneration from seedling explants	Bhuyan *et al.*, 1997
Paeonia suffruticosa	Plantlets from axillary buds	Beruto *et al.*, 2004
Pinus gerardiana	Shoot regeneration from cotyledon	Gupta *et al.*, 1994
Pinus strobus	Plant regeneration from adventitious shoot and mature embryos	Tang and Newton, 2005
Platanus acerifolia	Shoot regeneration from leaf explants	Liu and Bao, 2003
Populus deltoides	Somatic embryogenesis from stem containing axillary bud	Noel *et al.*, 2002

Prunus armenica	Plant let regeneration from immature embryo	Burgos and Ledbetter, 1993
Prunus avium	Somatic embryogenesis from Zygotic embryos	Garin *et al.*, 1997
Prunus avium	Multiple shoots from shoot tips	Hammatt and Grant, 1997
Prunus svium X *P. pseudocerasus*	Somatic embryos from rootstock	Pesce and Rugini, 2004
Quercus ilex	Somatic embryogenesis from leaf explants	Feraud and Espagnac, 1989
Quercus robur	Somatic embryogenesis from seedling stem and leaf explants	Cuenca *et al.*, 1999
Quercus suber	Somatic embryogenesis from mature leaf explants	Hernandez *et al.*, 2001
Q. serrata	Somatic embryogenesis from Immature zygotic embryos	Sasamoto and Hosoi, 1992
Syzygium cuminii	Multiple shoots from nodal segments	Jain and Babbar, 2003
Syzygium aromaticum	Multiple shoots from cotyledonary nodes	Mathew and Hariharan, 1990
Syzygium cuminii	Plantlet regeneration from epicotyl explants derived from seedlings.	Jain and Babbar, 2000
Tecomella undulate	Multiple shoots from nodal shoot segments	Rathore *et al*, 1991
Terminalia arjuna	Somatic embryogenesis from leaf callus	Kumari *et al.*, 1998
Theobroma cacao	Somatic embryogenesis from floral explants	Li *et al.*, 1998
Zizyphus mauritiana	Shoots from stem segments	Mathur *et al.*, 1995
Zizyphus jujuba	Plantlets from leaf explants	Gu and Zhang, 2005

Table 2.2. Some of the important micropropagated aquatic plants

Species	Explants	Authors
Anubias barteri	Plantlets obtained from leaf/petiole explants	Huang *et al.*, 1994
Aponogeton distachyus	Plant regeneration from shoot culture	Harder, 1968
Crassula hemsii	Shoot regeneration from leaf explants	Kane *et al.*, 1993
Cryptocoryne lucens	Plantlets from defoliated leaf	Kane *et al.*, 1990.
Cryptocoryne wendtii	Plants from aerial leaf explants	Kane *et al.*, 1999
Dionaea muscipula	Plantlets from rhizome tips	Parliman *et al.*, 1982
Griffithelta hookeriana	High rate of seed germination	Vidyashankari, 1987
Indotristicha ramosissima	High rate of seed germination	Vidyashankari, 1988
Lemna gibba	Callus induction and plantlet regeneration from leaf frond Explants	Chang and Chiu, 1978; Moon and Stomp, 1997, Li *et al.*,2004.
Lemna minor	Callus induction and plant let regeneration from pond explants	Yamamoto, 2001
Limnophilla indica	Regeneration of whole plants from toot tips	Rao and Mohan Ram, 1981.
Myriophyllum aquaticum	Shoot organogenesis from internodal stem explants	Kane *et al.*, 1991
Myriophyllum heterophyllum	Direct adventitious shoot/root regeneration from aerial leaf	Kane and Albert, 1989
Neptunia oleracea	Shoot organogenesis from explants	Kakkar and Mohan Ram1986

Polypleurum stylsosum	Germination from undehisced fruits	Sehgal *et al.*, 1993
Proserpinaca species	Shoots from aerial leaf	Kane and Sheehan, 1988
Nymphaea 'Daubeniana'	Plantlets from leaf explants	Jenks *et al.*, 1990
Nymphoides indica	Indirect shoot organogenesis through petiole explants	Jenks *et al.*, 2000
Spirodela oligorrhiza	Plantlet regeneration through callus obtained from frond explants	Li *et al.*, 2004
Utricularia inflexa	High rate of germination seeds	Doreswamy and Mohan Ram, 1969

CHAPTER III: MICROPROPAGATION OF *ILEX KHASIANA*

Introduction

Ilex khasiana Hook F. (Aquifoliaceae) is a holly tree which is rare and endemic to Khasi Hills , Meghalaya , India and is confined to a few pocket areas of the region. This tree species is mainly used for Christmas decorations and has potential biomass to maintain healthy environment (or ecosystem) for hilly regions. The wood tree of this is also utilized in making of furniture. In nature the propagation of this tree through seed germination is a rare phenomenon as seeds fail to germinate because of incomplete and ill rudimentary embryos. Also, the viable seeds have prolonged periods of dormancy. Like other woody species, the propagation is a slow process and the plant regeneration through vegetative cuttings is not possible. Because of the rapid deforestation, urbanization and erosion, *I. khasiana* has become rare and endemic to this region (Nayar and Sastry, 1990).

Micropropagation holds great promise for rapid propagation of rare and endangered plants particularly for the species with reproduction problem and /or extremely reduced population. The tools of biotechnology are being increasing applied now for conservation of plants (Prance, 1997; Feijoo and Iglesias, 1998; Lynch, 1999). Owing to extreme rarity, vulnerability of *I. khasiana,* a protocol for rapid micropropagation was required to fulfill the demand of plants on large scale production and for its conservation. Following studies were conducted using different explants of *I. khasiana.*

- Direct shoot multiplication
- Callus induction
- Caulogenesis
- Rooting / field transfer

Materials and Methods

Season vs. Source of Explants

Various explants from 10-12 years old mature tree of *I. khasiana* were collected from Upper Shillong, Meghalaya. Explants like leaves, shoot tips, nodal stem segments were excised from young twigs in four different seasons, Spring (March -April), Summer (June-July), Autumn (October- November) and Winter (December-January). The percentage of contamination, phenolic exudation, survival and multiplication rate were recorded.

Micropropagation of *I. khasiana* was carried out using different explants from mature trees and seedlings origin. Cultures of leaves, shoot tips, nodal segments obtained from mature trees of *I. khasiana* were affected by the seasonal variation. The rate of shoot multiplication and development appeared to be more in the materials collected during June/July. The rate of shoot development gradually decreased during the period of October to January.

Choice of explants

a) Explants from mature trees

Explants namely shoot segments, apical buds, leaves were collected from trees growing in Upper Shillong, Meghalaya. These explants were washed in Teepol (2% v/v) for 15-20 min. Explants were further rinsed thrice in distilled water and further immersed in 70% ethanol for 15-30 sec, and rinsed 5 times prior to surface sterilization with 0.05-0.15% mercuric chloride ($HgCl_2$) or 15% sodium hypochlorite (NaOCl) for varying periods of time and rinsed 5-6 times with autoclaved double distilled water. The end parts of explants exposed to sterilant were trimmed and inoculated aseptically in test-tube of the size 25 x 150 mm (Borosil, India Ltd.) containing 15 ml of different media (viz. MS, LS, White, Nitsch) supplemented with 3% sucrose, auxins and cytokinins in different concentrations ranging from 0.5-2.5 mg/l both singly and combinations. The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C (1.06 kg/cm^2). All the cultures were maintained under culture conditions where temperature was maintained at 25± 2°C under cool white fluorescent light of 200 μ moles m^{-2} sec^{-1} intensity with 16 h photoperiod.

To over-come the problem of browning and death of cultures, various pre-treatments were used:-

-50 mg/l Ascorbic acid (AA)

-0.5% Polyvinylpyrrolidone (PVP)

-75 mg/l Citric acid (CA)

-2% Charchoal (CHAR)

-Chilling at 4^0 C for 2 h (CHIL)

-Serial transfer (2-4-8-12-24-48-96 h intervals)

-Distilled water (D H_2O)

Frequent serial transfer of explants directly on the fresh medium at an interval of 2,4,8,12,24,48 and 96 h was done to remove brown exudates. All the cultures were maintained at dark at 25 ±2°C for 1 week prior to subculture on MS medium containing different growth regulator combinations. Observations on browning and percent survival of explants were done after every 24 h.

b) Explants from seedlings

Berries of *I. khasiana* were collected from mature trees during the months of December-January. Seeds were separated from the pulpy berries and washed thoroughly using 5% teepol for 5-10 min and rinsed thrice by double distilled water. These were surface sterilized with 70% ethanol for 30-60 secs followed by 15% sodium hypochlorite solution for 15 min and four rinses with sterile distilled water. The seeds were inoculated in medium supplemented with auxins and cytokinins singly or in combination at different range of concentration (0.5-2.5 mg/l).

Explants such as shoot tips, axillary buds, nodal segments, roots were excised from *in vitro* grown seedlings. Exudation of phenolic substances was not a hurdle for these seedling explants and hence pre–treatments against phenolic exudation were not required. These explants were also inoculated and maintained in culture rooms as mentioned in case of the mature explants.

All the procedures were carried out in a clean inoculation room. Inoculation was carried out under laminar flow cabinet fitted with ultra violet irradiation. Required glassware and other culture instruments (scalpel blade, forces, needles etc.) were sterilized along with culture media at 121°C and instruments were flamed using rectified spirit before use. All cultures were maintained under culture conditions where the temperature was maintained at 25±2°C under cool white fluorescent light intensity 200 μ moles m^{-2} sec^{-1} lamps with 16 h photoperiod.

After the prior experiment with different media , MS basal medium was finally selected for the subsequent studies.

Raising and Maintenance of Aseptic Cultures

Different explants like apical buds, nodal stem segments containing one axillary bud were excised from 1-2 months old *in vitro* grown seedlings and 10-12 yrs old mature trees of *I. khasiana.* Sterilization and pre-treatments of antioxidants for explants from mature trees were carried out as described in the sterilization technique. These explants were inoculated on MS medium containing 3% sucrose and 0.8% agar supplemented with either auxin and cytokinin or two cytokinins viz., BAP + KIN in combinations. Each culture tube contained one explant containing one bud. A minimum of 16 replicates for mature trees and 6 for seedlings (as less explant was available) were taken. For control, the explants were cultured on basal MS medium without growth regulators. All the sets of cultures were incubated under the culture room conditions. Browning was determined when explants showed any degree of discolouration and / or the medium colour changed from white to brown in case of mature explant cultures.

After 4 weeks, explants with proliferating shoot clumps were transferred to 250-500 ml conical flasks each containing approximately 50-75 ml medium. Cultures were transferred to fresh medium every 4 weeks. Observation records on shoot number and shoot length were made after every 2-3 weeks of culture.

Rooting of Isolated Shoots

For root initiation, shoots measuring around 2-3 cm in length were excised from multiple shoot clumps and transferred to various media like MS basal salt without auxins as control, MS supplemented with auxins, ½ MS with auxins and White media with auxins at different concentration ranging from 0.5-2.5 mg/l. The incubation period for root initiation was 20 days of which the initial 6 days were total darkness. The shoots were maintained under the same culture conditions as described earlier. The records on percent rooting, root number and root length were made after 4 weeks of culture.

Establishment of Callus Culture and Caulogenesis

Leaf discs (measuring 2x4 mm), stem nodal segments and apical buds were used for the establishment of callus cultures and caulogenesis in *Ilex khasiana*. MS medium supplemented with different concentrations and combinations of auxins (2,4-D and NAA) and cytokinins (BAP and KIN) were used for callus induction, shoot buds and caulogenesis . The range of concentrations of growth regulators for this present investigation was 0.5 to

2.5 mg/l. The pH of the medium was adjusted to 5.8 before autoclaving. Callusing was evident after 4 weeks and induced calli were subcultured on to the fresh medium after every 4 weeks for multiplication. The conditions required for growth and development of calli were evaluated. After raising sufficient stock, the callus cultures were subjected to various combinations and concentrations of cytokinins and auxins. At least 40 cultures were raised for each treatment. Each treatment and all the experiments were repeated thrice.

The calli derived from nodal and leaf segments were transferred to MS medium containing BAP at different concentrations (0.1 to 2.5 mg/l) singly or in combination with IBA or IAA (0.1-2.5 mg/l) for further experiment on regeneration from callus. All cultures were incubated both in dark and at different light intensities of 16 h photoperiod for further morphogenetic responses of callus. Cultures were observed at weekly intervals. Small shoots differentiating directly from callus were detached from the parent tissue and transferred to MS medium containing lower concentration of BAP (0.5 mg/l) for elongation.

Multiplication and Rooting of Shoots

Shoots regenerated directly from callus were multiplied on MS medium supplemented with BAP (1.0 mg/l) + IBA (0.1 mg/l). For rooting, 4-5 cm long elongated shoots with 3-4 nodes were cultured on MS medium (at various salt strengths) incorporated with auxins. The percentage of shoot initiation, number of roots per explants and the root length were recorded after 3 weeks of culture.

Cultures for shoot multiplication and rooting were initiated in flasks containing 50 ml medium. All cultures were maintained in tissue culture incubation room at different conditions of growth. The initial dark treatment for cultures for 1 week was carried out and compared with that grown at 25±2°C, 50-60 % humidity with 16 h photoperiod. Experiments were repeated thrice and 20 cultures were raised for each treatment. Observations were recorded at weekly intervals and standard deviation of mean was calculated (indicated by ±).

Results

I. Direct multiple shoot bud induction

a) Explants from Mature Trees

Nodal and shoot tip explants collected during different seasons from 10-12 yrs old mature trees of I. khasiana produced undesirable characteristics of browning into the medium and subsequently led to death of explants. It was observed that inspite of pre-treatments with antioxidants, the explants failed to get established in the culture medium. Various pre-treatments to control browning resulted in a survival of 15- 20% of cultures, whereas the rest turned brown and caused death of tissue (Fig. 3.1).

Seasonal Effect on Culture Establishment

The contamination and establishment of explants were greatly affected by the season in which they were collected from mature trees growing in the field. Serious browning of explants was experienced from adult wild trees during the winter month (Fig. 3.2).

Materials and Methods

Seasons vs. Explant Source

The influence of seasons on explant plays an important role in the initiation and establishment of cultures in the media. So selection of suitable source of explants in appropriate season is a vital process for the success of *in vitro* clonal propagation. Various explants from *N. tetragona* aquatic herb were collected from Smit pond, Shillong, Megahlaya. Explants like leaves, rhizome buds, flower buds, pre-mature- or mature undehisced fruits were excised from the plants in four different seasons, Spring (April), Summer (July), Autumn (October) and Winter (December- January)

Surface Sterilization

Premature- or mature undehisced fruits, rhizome tips, petioles, leaf, flower buds were collected from the pond. These explants were washed first with 2% (v/v) teepol for 10 min and then with tap water, followed by 15% (v/v) sodium hypochlorite (NaOCl) solution for 10 min and rinsed 5-6 times with autoclaved distilled water. The explants were further treated with 0.01-0.15 % $HgCl_2$ for 10 min and rinsed thrice with double distilled water to remove all traces of the sterilants. These were then treated with 70% ethanol for 10-30 seconds.

Media Compositions and Conditions

Different media such as MS, ½ MS, LS, Nitsch, White's media were used. Out of the various media tested in the initial experiments, MS medium was found to be the most suitable and it was finally selected. Both liquid and semi solid media were used for initiating *N. tetragona* cultures.

b) Explants from *in vitro grown* seedlings

Seeds obtained during December- January were cultured on MS medium with different cytokinins (BAP, KIN, Zeatin) and auxins (IBA, NAA). The germination percentage was only 5-8%. Various explants like nodal, apical bud etc. were excised from developing seedlings for the induction of direct shoot regeneration. Among these explants, nodal segments were found responsive and induced maximum shoot buds (Table 3.1).

The nodal segments excised from *in vitro* seedlings cultured on MS medium containing cytokinins both BAP (2.0 mg/l) and KIN (1.0 mg/l) resulted in maximum response and number of shoots (Photoplate 3.2 A,B). In this treatment, 85 % of the nodal explants containing single axillary bud resulted in an average of 10.2 shoots per explant. However, at higher concentrations of both BAP (2.5 mg/l) and KIN (1.5 mg/l), the number of shoot buds and the percentage of explants giving response declined comparatively. The same was noticed when the concentrations of both the cytokinins was reduced to 1.0 mg/l BAP and 0.5 mg/l KIN. Incorporation of IBA in the medium in addition to either BAP or KIN alone resulted in a poorer response of the nodal explants taking from *in vitro* grown young plants. The response of the nodal explants taken from mature trees was much less on the same medium (Table 3.1). In this case the maximum of 40% explants gave response and resulted in the formation of optimal 8 shoots per explant. The shoots were observed to have discoloured and distorted leaves with stunted growth in this case. The overall response of the nodal explants from mature trees was not as promising as was observed from the explants taken from the young *in vitro* grown plants. With both BAP and KIN in the different media (MS, ½ MS, White), it was found that on MS full strength medium, maximum number of shoots were initiated in the concentration of 2.0 mg/l BAP and 1.0 mg/l KIN. However with the increase or decrease in the cytokinin concentration, the numbers of shoots formed declined. In control where no cytokinin was incorporated in the medium a single shoot was seen to have emerged (Fig. 3.5).

MS with different concentrations of BAP or KIN alone showed single shoot formation from nodal explant. Higher concentration of BAP or KIN alone had no positive influence on the shoot multiplication and produced only one or two shoots with stunted internodes. BAP or KIN (2.0 mg/l) alone with IBA (0.5mg/l) in the MS medium resulted in only 3-4 shoots/explant (Table 3.1). With the increase in the concentration of KIN (2.5mg/l) in combination with IBA (0.5mg/l) only 1-2 shoot/explant with callus growth at the base of explant were observed (Table 3.1; Photoplate 3.3 A).

Elongation of *in vitro* Shoots

To promote shoot elongation, shoots obtained from seedling explants were transferred to the same medium. The *in vitro* obtained shoots attained a height of 3 - 4 cm within 30 days of culture (Photoplate 3.2 A). For further elongation, the *in vitro* grown shoot buds were cultured on MS medium containing reduced concentrations of BAP (0.5 mg/l) + KIN (0.25 mg/l).

II Callus Initiation

MS medium was found to be more suitable than White's for callus induction. MS medium with half strength salts was suitable to reduce the phenolic oxidation and browning of explants but the percentage of callusing was very low. Out of different explants (leaf discs, nodal and apical buds), leaf discs were the best for callus initiation. Leaf explant derived from one-month-old seedlings was more responsive in the callus initiation. The age of leaf discs also influenced the initiation of callus. The leaf explants obtained from 15-days old seedlings were not favourable. The explants if obtained from 21 to 45 days were the best source of explants for the induction of callus. The initiation and establishment of callus was influenced by the type of growth regulators, its concentration and combination. A combination of cytokinin and auxin (KIN + 2,4-D) supported callus induction. Other auxins like IBA, IAA when used singly or in combination with cytokinin (KIN or BAP) were also not responsive for callus induction. Callus initiation was obsereved on leaf explants within 4 week of culture in the medium with higher level of auxin 2,4-D (2.0 mg/l) with cytokinin KIN (0.5 mg/l) (Table 3.2; Photoplate 3.5 A). However with the increase or decrease of 2.0 mg/l 2,4-D concentration in the medium resulted in low callus development. Moreover 2,4-D with BAP combination also initiated low yield of callus. The initial dark treatment of 7 days of explants was helpful in inducing callus formation. The effect of light intensity on callus development was found to be pronounced. The low light intensity of 50μ moles m^{-2} sec^{-1} was favourable to induce healthy callus. The increase of light intensity to 150-200 μ moles m^{-2} sec^{-1} resulted in hard and dark brown callus (Table 3.3; Photoplate 3.5 A).

Orgnogenesis or Caulogenesis from Callus.

With the aim of inducing shoot bud organogenesis and subsequent plant regeneration, the callus obtained from leaves were transferred to ½ MS medium with different concentrations of cytokinins and auxins at a range of concentrations from 0.5-2.5 mg/l. In the concentration of 1.5 mg/l BAP and 0.5 mg/l IBA in ½ MS medium, the callus resulted in shoot bud proliferation, of average 12 shoots per leaf disc callus and the response was 60 % (Table 3.4). If the concentration of BAP was decreased to 0.5 mg/l with 0.5 mg/l IBA, the callus resulted in less number of shoot buds in an average of 4 per leaf disc callus and the percentage of response of shoots was declined. In the same way, the decrease of number of shoot buds was observed when the concentration of BAP was increased to 2.5 mg/l (Table 3.4). However, when MS full strength medium was taken with the same concentration and combination of above growth regulators, the number of shoot buds from the callus was not promising.

***In Vitro* Rooting**

Sufficient grown shoots from *in vitro* formed shoot clumps were carefully excised and transfered on ½ MS and full strength MS salts supplemented with IBA (0.5-3.0 mg/l), IAA (0.5 – 3.0 mg/l) and NAA (0.5 – 3.0 mg/l) for root induction. In all the cases root initiation started within 2 weeks of culture. Out of the three auxin tested, IBA was effective in inducing roots. About 90% excised shoots developed 2-4 roots/shoot within 15 days of culture on ½ MS fortified with IBA at an optimal concentration of 2.0 mg/l (Table 3.5; Fig. 3.6). IBA improved the rooting percent, root quality and number of roots. The increase of IBA concentration reduced the formation of roots.

On full strength MS medium, excised shoots showed a reduction on *in vitro* root formation in *I. khasiana* with IBA treatments. Rhizogenic activity was reduced to 50% on full strength MS when compared to ½ strength MS which was 80%.

Initial dark incubation of root inducing cultures of ½ MS medium with IBA (2.0 mg/l) was quite promising. Rooting was observed within 15 days and the percentage of rooting was 90% with root quality and root number 5-6 roots/shoot (Fig. 3.7; Photoplate 3.8 A).

Discussion

The rapid depletion of world's biological wealth, especially important forest species, is one of the most serious global crises today. Although extinction is a natural process, in the recent past, this phenomenon has been drastically augmented by the indiscriminate interference by man on the natural ecosystem upsetting the ecological equilibria. The wild natural resources of forest trees are already depleted and extinction of rare species and erosion of gene pool are the problems now alarming the natural ecosystem.

Conservation of gene pool is not a new concept of modern days, and it is a rediscovery and renovation of modern days. Realising the importance of the factors now facing the serious erosion of 'gene pool' and imbalance of ecosystem, the present study was undertaken for mass propagation and conservation of *I. kahsiana* and the results obtained are discussed in the light of relevant literature. The present work mainly aimed at establishment of direct shoot multiplication, callus cultures and direct regeneration or caulogenesis and germplasm conservation.

In general, woody taxa are difficult to regenerate through micropropagation, but successful reports exist for the micropropagation of few tree species. Although some tree taxa can be micropropagated from tissues collected from mature trees, many others could propagate only from juvenile tissues i.e., embryos or seedlings. However, micropropagation through mature tree explants is usually preferred over embryos or seedlings because it is possible to maintain the genetic stability to maintain the desired qualities later. Clonal propagation from explants of mature trees, if at all possible, may require a cultural regime that is considerably different from that required for propagation from embryo explants.

Explant browning and media staining were the major obstacles in the initial establishment of cultures when nodal segments of *I. khasiana* from mature trees were used in the medium. The medium turned brown within 24 h of culture and the explants died due to release of brown exudates. Similar problems during primary establishment of cultures have been attributed to the existence of large quantities of polyphenolic compounds in the tissues of woody species (Hu and Wang, 1983; Dhar and Upreti, 1999; Chang *et al.*, 2001). Browning of cultures has been reported to have occurred through action of copper containing enzymes such as polyphenols oxidases and tyrosinase (Lerch, 1981). The oxidation of polyphenols by polyphenolic oxidases is reported to inhibit enzyme activity, by killing the explants. Similar problems of browning of cultures were reported in Teak (Gupta *et al.*, 1980); *Bauhinia vahlii* (Dhar and Upreti, 1999). To overcome this problem, a wide range of pre- treatments of explants were adopted for overcoming browning of cultures. Various pretreatments given to the explants were with ascorbic acid, PVP, citric acid, charcoal, chilling and serial transfer of explants on to the fresh medium. Out of various pretreatments, PVP and charcoal in the medium proved to be useful for preventing browning of tissue cultures of *I. khasiana* where the survival rate of explants was below 30%. But injurious effect of exudates from plant on the establishment was avoided by serial transfer of explants to fresh MS medium supplemented with 0.5% PVP at an interval of 2,4,8,12,24,48,96 h. Following this procedure 50% of the explants could be survived. This is in agreement with the earlier results obtained by transferring explant repeatedly onto fresh medium at regular intervals (Lloyd and McCown, 1980; Bhatt and Dhar, 2000). Although pretreating explants with PVP (Walkey 1972; Mathew, 1993), distilled water (Vietez and Vietez, 1980), PVP + sucrose, antioxidant solution (Anderson , 1975; Ziv and Halvy, 1983), sucrose (Gupta *et al.*, 1980), chilling at 4°C (Dhar and Upreti, 1999) have been found to be helpful in reducing phenol induced browning in a number of plant species, in the present study these treatments showed poor establishment of *I. khasiana* mature explants. Tissue browning could be reduced by maintaining the shoot cultures in darkness for an initial period of one week prior to subculture. Similar findings were recorded in guava when the cultures were incubated in dark (Amin and Jaiswal, 1987). According to Hu and Wang (1983), the reduced light intensity is beneficial for reducing oxidation.

It is fairly well established that successful establishment of aseptic cultures of field grown trees is influenced by the different seasons. In the case of *I. khasiana*, mature explants were seasonally effected. During summer (June-July), a relatively low rate of browning of shoot was recorded. In the autumn season (Ocotber-November), browning of shoot explants was higher than summer. Winter season (Dec-January), recorded the highest browning and contamination of the explants. Similar effects of season on bud sprouting and explant browning have also been reported in Teak (Gupta *et al.* 1980) and *Acacia* (Detrez, 1994).

The regeneration of plantlets from explants collected during spring (April) was comparatively low as compared to those collected in summer (June–July). During autumn season, low percentage in establishment of cultures was observed. The responce of explant was significantly less when collected during winter (Dec.-Jan.). A comparatively low rain-flush in spring and fall season than the regular rains during summer may be one of the causes in the performance of culture establishment. During winter, the lowest survival and performance of explants of shoots in *I. khasiana* was observed and the maximum phenolic exudation and death tissues resulted. Similar reports exist, where the dormancy of winter buds was more difficult to break in the cultures of tree species *Acacia* (Detrez, 1994), *Eucalyptus* (Chang *et al.*, 1992), *Sequoia* (Boulay, 1987).

Although the performance of shoot explants collected during spring was comparatively less than the summer, spring explant cultures performed better in the long run than the summer ones.

Browning of the cultures was not a problem when the explants were excised from *in vitro* grown young seedlings of *I. khasiana*. Hence no pre-treatments were required in this case. Yadav *et al.*, (1990) and Salvi *et al.* (2001) also reported that seedlings proved to be the best source of explants than adult ones for controlling browning in *Syzigium cumini* and in *Azadirachta indica* respectively.

The rate of contamination coupled with browning in *Ilex* was found more serious from the explants collected from mature trees. Another problem encountered in the explants from mature trees was the appearance of necrotic spots in later part of the culture establishment. Few plants which were established failed to evoke satisfactory response. The multiple bud induction was obtained from nodal explants of mature trees and *in vitro* grown young seedlings on MS medium supplemented with different concentrations of auxins and cytokinins. The highest number of multiple shoots per explant resulted from nodal explant of young seedling. More than 10 buds arose from the single node stem of seedling origin in MS medium supplemented with cytokinins BAP (2.0 mg/l) and KIN(1.0 mg/l) in combination, whereas the number of shoots obtained from mature explant was 8 shoots per explant under the same conditions of cultures. Salvi *et al.* (2001) reported that comparatively high regeneration potential was exhibited by seedling explants than the explants obtained from mature trees in *Azadirachta inidca*. Das *et al.* (1996) reported that excessive phenolic oxidation prevented induction of multiple shoot formation in trees of cashew, therefore juvenile seedlings were used as the source for *in vitro* studies. Similar observation was reported by Mathew (1993) where seedling culture of clove was more suitable than explants from mature trees. Rao and Sita (1996) reported that the success of *in vitro* shoot or root formation is often related to maturity or age of the donor plant. It has also been established in many herbaceous plants that younger explants exhibit greater morphogenetic potential than older plants (Yepes and

Aldwinckle, 1994; Nikam and Shilole, 1999), as they might have more metabolically active cells with hormonal and nutritional conditions that are responsible for increased organogenesis (Famiani *et al.*, 1994). Dkurkovic (2003) reported high rate of *in vitro* multiple shoots and plant regeneration from juvenile axillary buds whereas mature plants failed to regenerate.

Propagation through axillary bud multiplication is an easy and safe method for obtaining uniformity and it also assures the consistent production of true –to-type plants within a short span of time (George; 1993; Salvi *et al.*, 2001). However, factors such as poor growth (Linington, 1991; Durkovic, 2003), excessive phenolic oxidation (Linington, 1991; Bhatt and Dhar 2000), difficulty in rooting (Bonga, 1977, Noiton *et al.*, 1992) have been shown to limit *in vitro* culture of woody plants. Several studies have been carried out to optimize conditions for the *in vitro* regeneration and multiplication of woody species. In general woody taxa are difficult to regenerate under *in vitro* conditions, but success have been achieved in tree species like *Tabebuia* sp. (Rajani and Urs, 1998), *Acacia mangium* (Bhaskar and Subhash, 1996), *Santalum album* (Sita, 1986), *Eucalyptus* (Mascarenhas and Muralidharan, 1989), *Azadirachta indica* (Chaturvedi *et al.*, 2004), *Coffea arabica* (Naidu and Sreenivasan, 2004) and *Paeonia suffruticosa* (Beruto *et al.*, 2004).

In the present study, maximum explants were established in MS medium. Successes with plant cell, tissue and organ cultures have been reported to depend on the use of suitable media. The composition of culture media is one of the most important criteria for the successful establishment of cultures (McCown and Sellmer, 1987). Brown *et al.* (1995) reported that about half of the embryo induction media used across all the plant species are MS based media. Similar superior effects of MS medium over the other media in producing multiple shoots were also reported in woody species like *Fragaria indica* (Bhatt and Dhar , 2000), *Platanus acerifolia* (Liu and Bao, 2003) and *Zyziphus jujube* (Hossain *et al.*, 2003).

Out of the various cytokinins tried (BAP, KIN, Zeatin), the combination of BAP and KIN proved to be essential for multiple shoot formation in *I. khasiana*. Axillary branching in shoot tip cultures occurred only when exogenous supply of cytokinins was given. It is well documented that BAP stimulates axillary shoot multiplication in many plant species (Devi *et al.*, 1994; Lakshamanan *et al.*, 1997; Sahoo and Chand, 1998;Sansberro *et al.*, 1999; Panai et al., 2000; Beena *et al.*, 2003). In the present study, the combination of (2.0 mg/l) BAP + (1.0 mg/l) KIN was found optimal for the induction of 10-11 shoots buds/explant. Kang *et al.* (1994) reported that BAP in the medium was beneficial in *Fragaria xananassa*. Similar studies were also reported by Babu *et al.* (2003), where BAP and KIN incorporated together in WPM medium initiated 20 shoots/single shoot in camphor tree (*Cinnamomum camphora*). Increasing the concentration of BAP and KIN in combination in the medium reduced number of shoot production. The suppressive effect of higher cytokinin concentration has been reported in some woody species (Amin and Jaiswal, 1993; Bhatt and Dhar, 2000), and *Saussarea obvallata* (Joshi and Dhar, 2003). Stunted growth in *I. khasiana* resulted in the medium containing higher concentration of BAP.

The incorporation of auxin at low concentration with cytokinin was found to be necessary for the growth and development of emerging buds in *Ilex*. The lower auxin and higher cytokinin ratio in shoot bud differentiation was well documented in *Nicotiana* species (Skoog and Miller, 1957). Out of the different auxins used, IBA in medium was found more effective with an enhanced shoot elongation in *I. khasiana*. Martin (2003) also reported an enhanced shoot multiplication with combination of both BAP and IBA in *Rotula aquatica*. Babu *et al.* (2003) also found that adding IBA in low concentration along with BAP and KIN in combination induced better shoot multiplication rate of explant on WPM medium. Similar results in a herbaceous plant *Ceropegia candelabrum* were obtained by Beena (2003).

Previous reports on micropropagation of *Ilex* species using BAP alone on the medium showed initiation of a low number of average shoots /explant. Morte *et al.* (1992) reported that axillary buds from 10-15 yrs old tree of *I. aquifolium* yielded 2 axillary buds/explant on medium supplemented with BAP alone. Sansberro *et al.* (1999) also obtained an average of 4 shoots/explant from nodal segments of 2 yrs old plant. The low number of average shoots in *Ilex* species reported earlier could be due to the age factors of the plants. In our present studies 15-45 days young seedlings were used as the sources of explants where a total of about 10 shoots/explant were formed in the MS medium supplemented with 2.0 mg/l BAP in combination with 1.0 mg/l KIN.

Elongation of *in vitro* shoots of *Ilex* was not markedly enhanced by the addition of GA_3 at various concentrations. Similar effect of GA_3 had been reported on the shoot multiplication of *Symonanthus bancroftii* (Panai *et al.*, 2000). Sahoo and Chand (1998) reported a synergestic effect in shoot elongation, on multiple shoot formation and internode elongation of *Tridax* in medium containing GA_3 in combination with BAP.

In the present finding, higher callus was induced from leaf discs cultured in MS medium containing the combination of auxin 2, 4-D (2.0 mg/l) and KIN (0.5 mg/l). Similar result using 2, 4-D with KIN has been reported in *Hypericum perforatum* leaves (Pretto and Santarem, 2000). 100% callus induction on MS medium supplemented with 2, 4-D and KIN was reported from different explants of *Acacia mangium* (Xie and Hong, 2001). A positive effect of a short inductive treatment of 2, 4-D application has been reported for apple (Yancheva *et al.*, 2003). Callus induction and proliferation systems are known to be very useful for the study of biosynthesis of natural products and the factors that influence it, giving some possibilities of controlled production. This approach has been used in the plant species viz., *Hypericum* and *Centaurium* spp. (Ferrari *et al.*, 1999; Schmidt *et al.*, 2000). Culturing the calli on ½ MS medium supplemented with BAP (0.5 mg/l), with lower 2,4-D (0.5 mg/l) or gradual removal of 2, 4-D resulted in shoot induction in four weeks time.

A period of dark treatment promoted adventitious shoot bud formation from callus of *I. khasiana,* however, further development of shoot was required by light treatment. Shoot formation was the best when leaf explants were cultured for 20 days in the dark. By increasing the duration of darkness to 30 days, more calli and fewer shoots were achieved. Arzate –Fernandez *et al.* (1997) reported that the degradation of auxins in the media is known to occur more quickly under light conditions than under dark conditions. It may be possible that exposure to a continuous dark culture period may modify the proportion of endogenous cytokinins and auxins, resulting in a stimulation of shoot formation on leaf explants of *I. khasiana.*

Keeping the callus in dark period up to 30 days or low light intensity (50-75 µmoles m^{-2} sec^{-1}) influenced the rate of shoot multiplication. This result is in agreement with that of Korban *et al.* (1992) and Miguel *et al.* (1996). They reported that dark incubation or low light intensity can affect and stimulate organogenesis by increasing endogenous levels of indole-acetic acid. This hormone, known to influence cell division and differentiation, interacts with exogenously supplied cytokinin and auxins for shoot induction.

The age of the explants plays a vital role in the regeneration of tissue culture experiments (Murashige, 1974; Gamborg and Phillips, 1996; Lin *et al.*, 1998; Molina *et al.*, 2002). The leaf disc explants used from 15-45 days old seedlings gave comparatively high yield of callus. Gu and Zhang (2005) reported 10 day old leaves showed better yield of shoots in *Zizyphus jujube.* Similar report was also obtained by Carneiro *et al.* (1999) in *Neorgelia cruenta* cultures. Joshi and Dhar (2003) also reported that 10-15 days old seedling explants regenerated maximum callus and shoot formation on MS medium containing BAP and NAA in *Saussurea obvallata.* It has already been established that younger explants exhibit greater morphogenetic potential than older explants (Welander, 1988; Fasolo *et al.*, 1989; Yepes and Aldwinckle, 1994). This could be attributed to the presence of more metabolically active cells with hormonal and nutritional conditions that are responsible for increased organogenesis (Famiani *et al.*, 1994).

Culturing the calli on ½ MS medium incorporated with BAP (1.5 mg/l) resulted in more than 60% shoot induction. Both KIN and Zeatin in the medium resulted in lower percentage of shoot formation. The gradual removal of 2, 4-D from the medium was necessary for the initiation of shoot buds. Such results have been reported by Kang *et al.* (1994) who found BAP as the most suitable growth regulator for producing multiple shoots from leaf explants in *Fragaria ananassa.* Pretto and Santarem (2000) also found that shoot induction from cultured callus could be enhanced in medium containing BAP alone. However, in contrast, Yang *et al.* (2001) reported that explants cultured on medium supplemented with BAP developed either extensive callus or distorted shoots that failed to proliferate. On the other hand Gentile (2002) found the stimulatory effect of BAP on induction of adventitious shoots from leaves of *Prunus persica.*

The most difficult stage of regeneration in woody species is the induction of roots on new shoots (Bonga, 1977). The concentration of growth regulators and its presence in the medium plays an important role in the rooting system. The determination of root induction usually depends on the auxin or auxin/cytokinin ratio in the medium. High concentration of auxin has been reported to induce roots or callus from different explants (Skoog and Miller, 1957; Arora and Bhojwani, 1989; George, 1993).

Root initiation was observed form 2nd week onwards following the transfer of the shoots to the rooting medium. Results were different based on the concentrations of IBA as well as strength of basal medium used. Out of the different basal media tried, 90% rooting was observed in shoots cultured on ½ strength MS with 2.0 mg/l IBA. Rooting decreased with the increase of IBA concentration. 5-6 roots measuring about 4 cm length were found to have initiated in the same cultures kept in dark for 7 to 10 days. Half strength MS medium with IBA was used to induce rooting in *Vicia pannonica* by Sancak *et al.,* 2000. Yang *et al.* (2001) also reported that individual elongated shoots of *Swainsona salsula* were rooted on ½ MS medium supplemented with 2.0 mg/l IBA. George (1996) also reported a lower mineral content to be more suitable for *in vitro* rooting of woody species. Similar result of using ½ MS was reported in *Lonicera tatarica* by Palacios and Leech (2002). Bhatt and Dhar (2000) also reported the use of half strength MS salt with NAA for rooting in the *in vitro* excised shoots of Indian wild strawberry. Similar reports using IBA on rooting have been reported in several plant species (Jusaitis, 1997; Caboni and Tonelli, 1999; Wawrosch *et al.,* 2001, Joshi and Dhar, 2003).

Table 3.0.Composition of some plant tissue culture media (Composition mg/l)

Components	White's	MS	LS	B_5	Nitsch
Macronutrient					
NH_4NO_3	-	1650	1650	-	720
KNO_3	80	1900	1900	2527.5	950
$CaCl_2.2H_2O$	-	440	440	150	-
$CaCl_2$	-	-	-	-	166
$MgSO_4.7H_2O$	750	370	370	246.5	185
KH_2PO_4	-	170	170	-	68
$(NH_4)_2.4H_2O$	-	-	-	134	-
Ca $(NO_3)_2.4H_20$	300	-	-	-	-
Na_2SO_4	200	-	-	-	-
$NaH_2PO_2.H_2O$	19	-	-	150	-
KCl	65	-	-	-	-
Micronutrient					
KI	0.75	0.83	0.83	0.75	-
H_3BO_3	1.5	6.2	6.2	3	10
$MnSO_4.4H_2O$	5	22.3	22.3	-	25
$MnSO_4.H_2O$	-	-	-	10	-
$ZnSO_4.7H_2O$	3	8.6	8.6	2	10
$Zn.Na_2$.EDTA	-	-	15	-	-
$Na_2MoO_4.2H_20$	-	0.25	0.25	0.25	0.25
MoO_3	0.001	-	-	-	-
$CuSO_4.5H_20$	0.01	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	-	0.025	0.025	0.025	-
$Fe_2(SO_4)_3$	2.5	-	-	-	-
$Fe_2SO_4.7H_20$	-	27.8	27.8	-	27.8
Na_2.EDTA.$2H_2O$	-	37.3	37.3	-	37.3
Sequestrene 330Fe	-	-	-	28	-
Organic constituents					
Inositol	-	100	100	100	100
Nicotinic acid	0.05	0.5	-	1	5
Pyridoxine HCl	0.01	0.5	-	1	0.5
Thiamine HCl	0.01	0.1	0.4	10	0.5
Glycine	3	2	-	-	2
Folic acid	-	-	-	-	0.5
Biotin	-	-	-	-	0.05
Sucrose	2%	3%	3%	2%	2%

Optional constituents of Linsmaier and Skoog:
Aminobenzoic acid 0.1 mg/l, GA_3 1.0 mg/l, Adenine sulfate 40 mg/l, Guanylic acid 200 mg/l, L-Glutamine 500 mg/l, Tyrosine 100 mg/l, Casein hydrolysate 1-3 g/l, Cytidylic acid 200 mg/l and L-Asparangine 500 mg/l.

Table 3.1. Effect of different concentration of cytokinins and auxins on direct shoot multiplication of nodal stem explants derived from young seedlings or mature trees

Explants	**Growth regulators (mg/l)**			**% Nodal explants regenerating shoots**	**Average shoot number per explant**	**Relative Callus growth**
	Cytokinins		Auxin			
	BAP	KIN	IBA			
Young plants	0.5	--	0.5	30	1.0 ± 0.4	-
	1.0	-	0.5	50	2.6 ± 1.10	-
	2.0	-	0.5	60	3.9 ± 1.2	-
	2.5	-	0.5	50	2.4 ± 1.05	-
	-	0.5	0.5	30	1 ± 0.5	-
	-	1.0	0.5	40	1.4 ± 0.4	+
	-	2.0	0.5	50	3.6 ± 1.02	++
	-	2.5	0.5	40	1.6 ± 0.5	++
	1.0	0.5	-	60	6.5 ± 1.55	-
	2.0	1.0	-	85	10.2 ± 1.40	+
	2.5	1.5	-	70	7.0 ± 1.45	-
Mature trees	0.5	-	0.5	20	1 ± 0.5	-
	1.0	-	0.5	40	1.4 ± 0.55	-
	2.0	-	0.5	50	1.0 ± 0.4	-
	2.5	-	0.5	40	1.0 ± 0.60	-
	0.5	-	0.5	20	1.0 ± 0.5	-
	1.0	-	0.5	40	1.4 ± 0.55	-
	2.0	-	0.5	50	1.0 ± 0.4	-
	2.5	-	0.5	40	1.0 ± 0.60	-
	-	0.5	0.5	10	1.0 ± 0.5	-
	-	1.0	0.5	30	1.2 ± 0.55	+
	-	2.0	0.5	40	1.0 ± 0.60	++
	-	2.5	0.5	20	1.0 ± 0.40	+
	1.0	0.5	-	20	5.5 ± 0.60	-
	2.0	1.0	-	40	8.0 ± 0.5	+
	2.5	1.5	-	20	5.0 ± 0.80	-

± SD = Standard Deviation

Data recorded after 4 weeks

Data represents an average of 10 explants per treatment

Table 3.2. Effect of 2,4-D and cytokinins (BAP/KIN) on callus development from leaf discs of *I. khasiana* after 4 weeks of culture

MS + Growth regulators (mg/l)			**% of callus initiation**	**Intensity of callus development**	**Nature of callus**
Auxin	Cytokinins				
2,4-D	BAP	KIN			
0.5	0.1	-	30	+	White and poor callus
1.0	0.5	-	40	+	Light brown and poor callus
1.5	1.0	-	50	++	White callus
2.0	0.5	-	60	++	Brown callus
2.5	1.0	-	40	+	Hard and poor callus
0.5	-	0.1	40	+	White callus
1.0	-	0.5	60	+	White callus
1.5	-	1.0	70	++	Shining and light brown callus
2.0	-	0.5	80	+++	Healthy callus
2.5	-	1.0	60	++	Hard with dark brown areas

Data represents an average 10 explants per treatment;

Callus induction was initially done in dark period (6-10) days then 16 h photoperiod at 25±2°C.

+ poor callus; ++ medium; +++ profuse callus

Table 3.3. Effect of light intensity on productivity of callus from leaf discs of *I.khasiana* on MS medium + 2, 4-D (2.0 mg/l) + KIN (0.5 mg/l)

Intensity of light (μ mols m^{-2} s -1)	**Callus growth after 6 weeks**	**Type of callus**
0 - 5	+	White callus
50	+++	More profuse and healthy callus
100	++	Shiny and light green
150	++	Hard with dark brown areas
200	+	Hard yellow with shiny areas

'+' denotes low growth of callus

'++' denotes medium growth of callus

'+++' denotes high growth of callus

Table 3.4. Effect of various combinations of cytokinins and auxins on morphogenetic responses of *I. khasiana* calli after 30 days of culture.

Basal medium	**Growth regulators (mg/l)**			**Mean number of shoot buds ± SD**	**Percentage of shoots**
	BAP	KIN	IBA		
½ MS	0.5	-	0.5	4 ± 0.55	40
	1.0	-	0.5	6 ± 0.84	50
	1.5	-	0.5	12 ± 0.54	60
	2.0	-	0.5	8 ± 1.40	50
	2.5	-	0.5	6 ± 0.72	40
	-	0.5	0.5	1.5 ± 0.47	10
	-	1.0	0.5	2 ± 0.72	20
	-	1.5	0.5	4 ± 0.84	30
	-	2.0	0.5	3 ± 1.50	40
	-	2.5	0.5	2 ± 0.78	30

± SD

Data represents an average of 10 explants per treatment

½ MS - half strength Murashige and Skoog medium

Table 3.5. Root formation on the *in vitro* derived shoots of *I. khasiana* on ½ MS basal salt medium containing different auxins after 4 weeks of culture

Auxins	**Concentration (mg/l)**	**Percentage of root formation**	**Mean number of shoots per explant**
IAA	0.5	10	1.5 ± 1.2
	1.0	20	2.6 ± 0.95
	2.0	40	3.4 ± 0.85
	2.5	30	2.0 ± 0.60
IBA	0.5	40	2.6 ± 0.60
	1.0	65	4.58 ± 1.20
	2.0	90	5.60 ± 1.60
	2.5	60	5.00 ± 1.02
NAA	0.5	20	1.5 ± 1.05
	1.0	30	1.8 ± 1.08
	2.0	50	3.8 ± 1.56
	2.5	40	2.9+1.42

± SD

Control: 1 - 2 roots developed in ½ MS basal salt medium

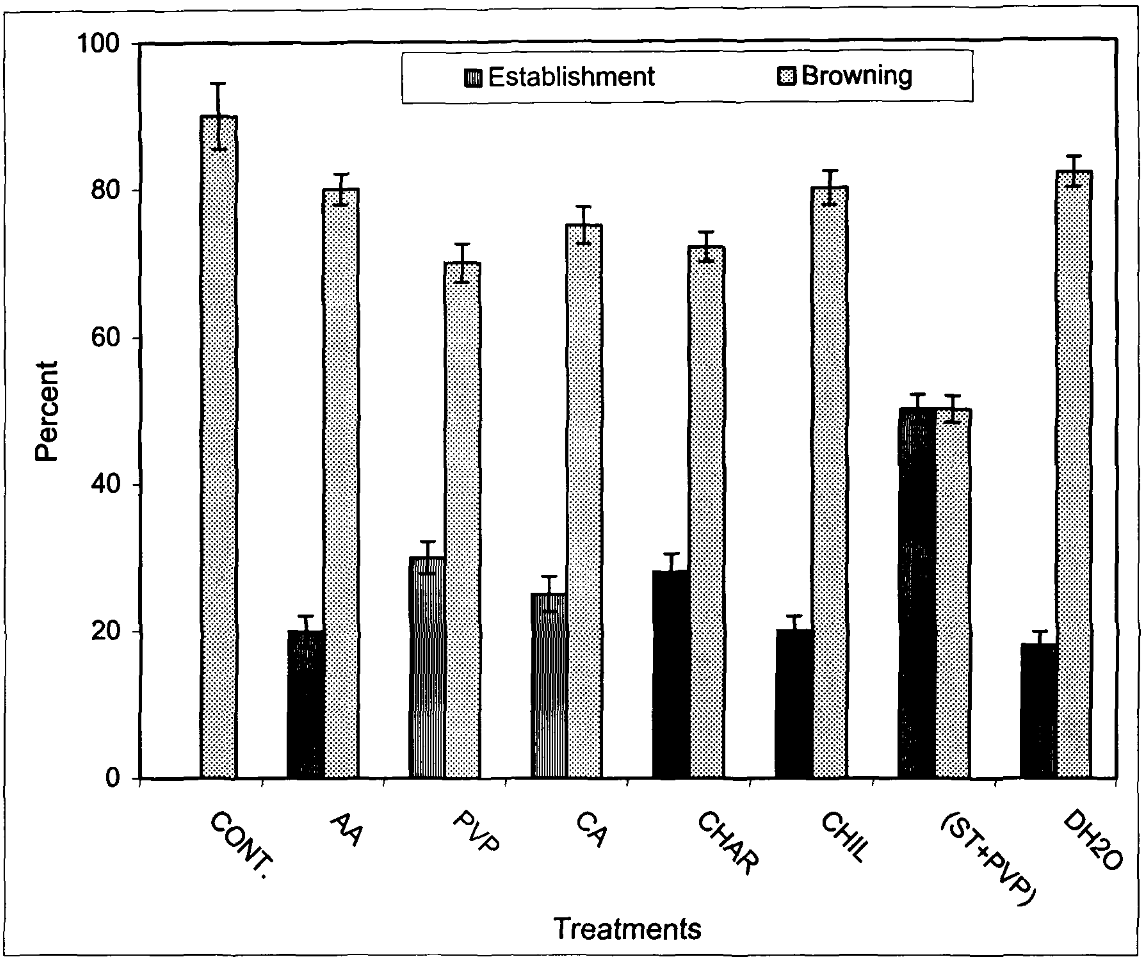


Fig. 3.1. Effects of various pre-treatments in preventing browning of mature tree explants of *I. khasiana*. CONT–Control, AA–Ascorbic acid (50 mg/l), PVP–Polyvinyl pyrrolidone (0.5%), CA-Citric acid (75 mg/l), CHAR-Charcoal (2%), CHIL– Chilling (at 5^0C for 2 h), ST + PVP–Serial transfer (2-4-8-12-24-48-96 h intervals) and DH_2O–Distilled water

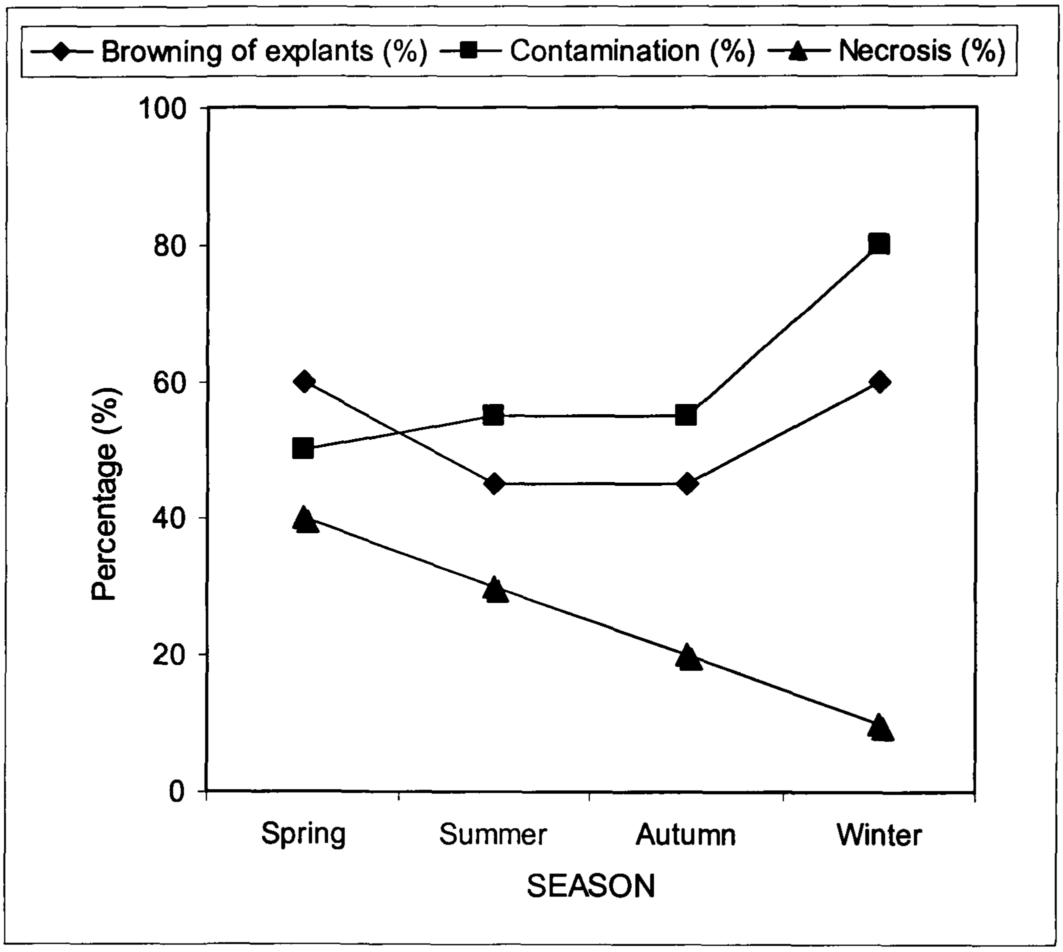


Fig. 3.2. Effect of season on cultures of nodal stem explants from mature trees of *I. khasiana* inoculated

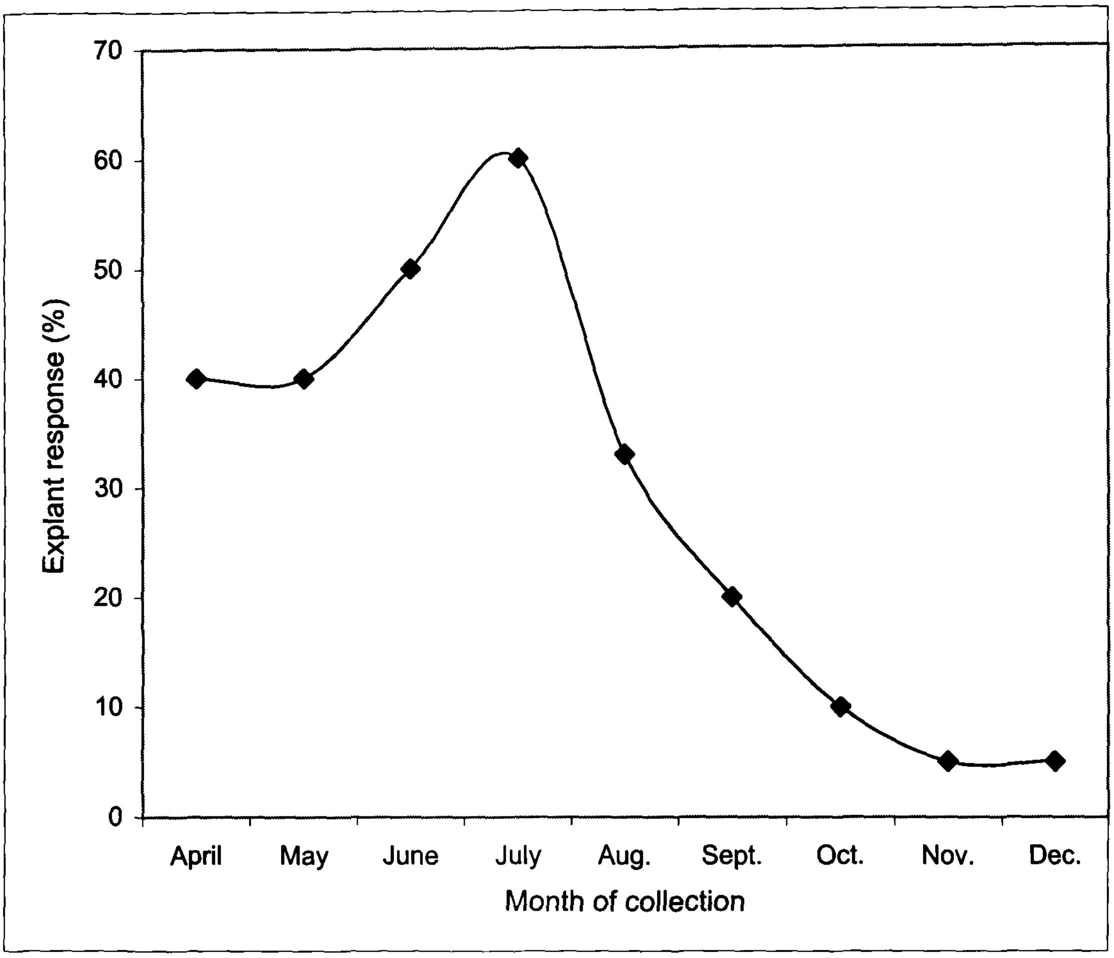


Fig. 3.3. Influence of the season on bud break from the nodal explants of *I. khasiana* inoculated in MS basal medium

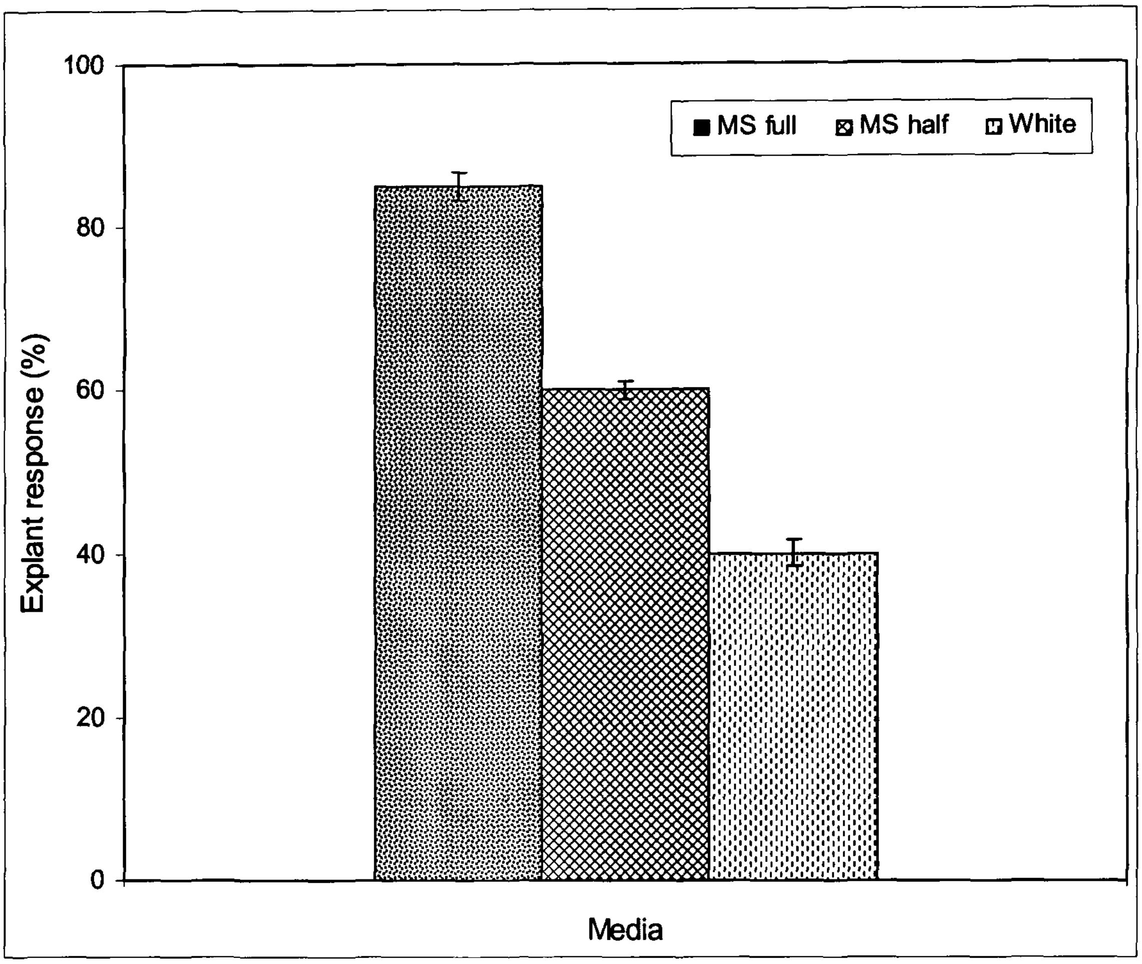


Fig. 3.4. Effect of different media on the percentage of explant response in *I. khasiana*

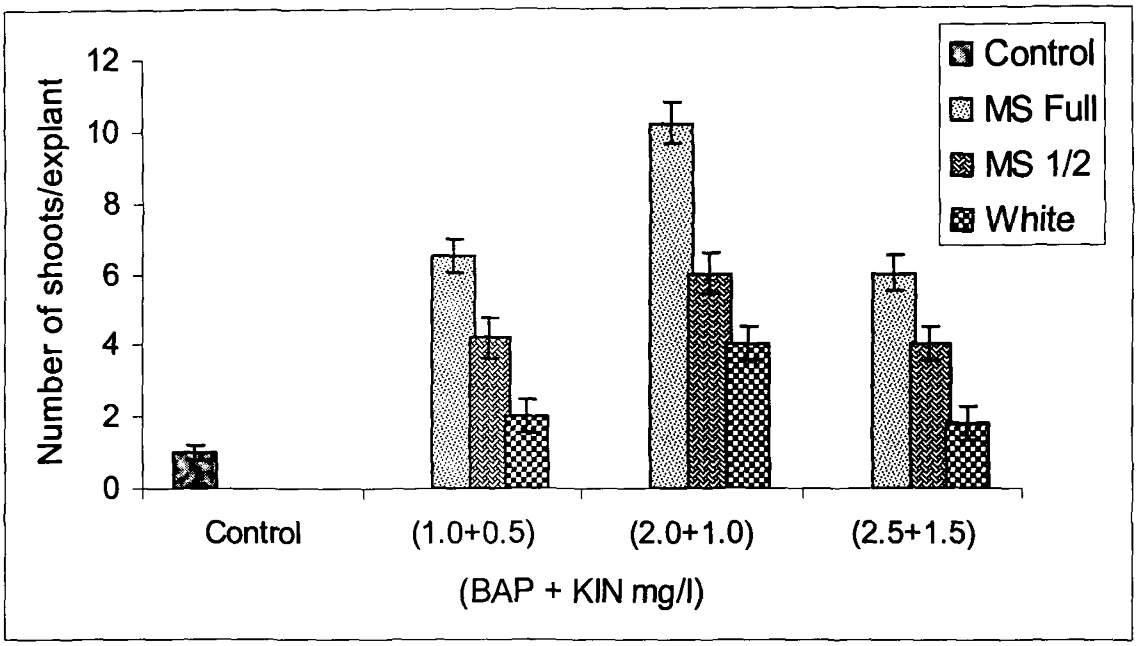


Fig. 3.5. Effect of various concentrations of cytokinins (BAP+ KIN) on the number of shoots from nodal stem segments after 45 days of culture

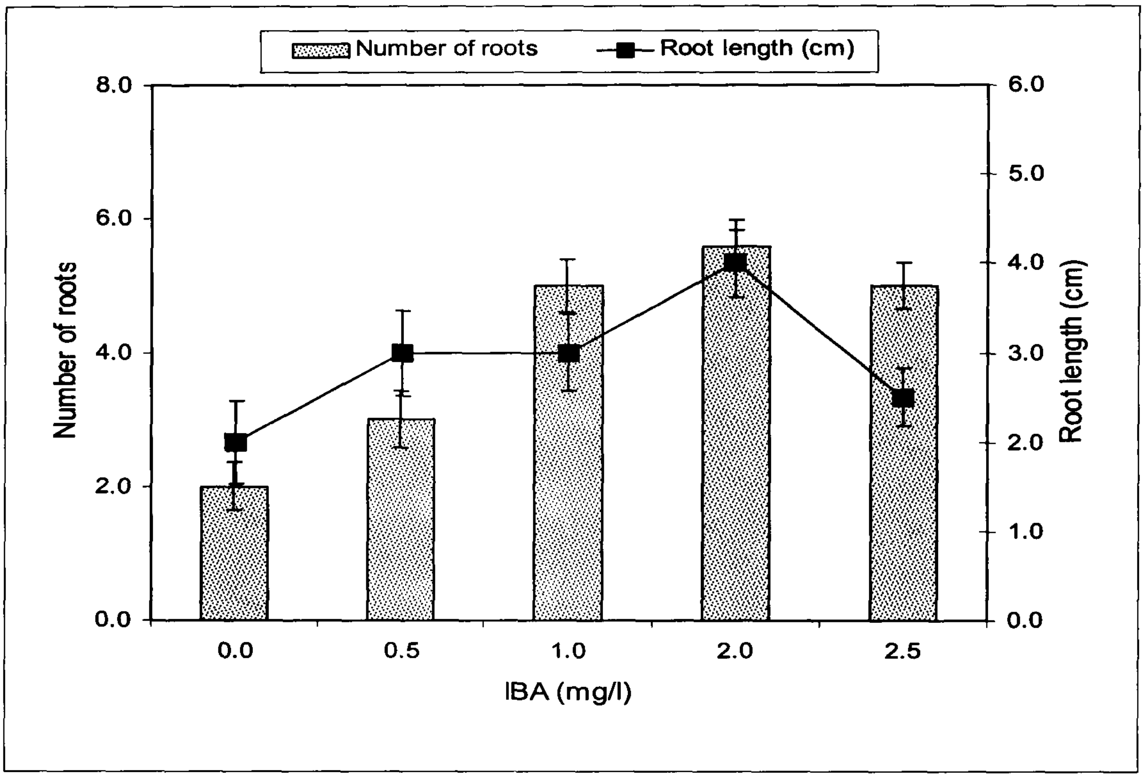


Fig. 3.6. Effect of different concentrations of IBA on root multiplication and elongation from *in vitro* derived shoots after 4 weeks

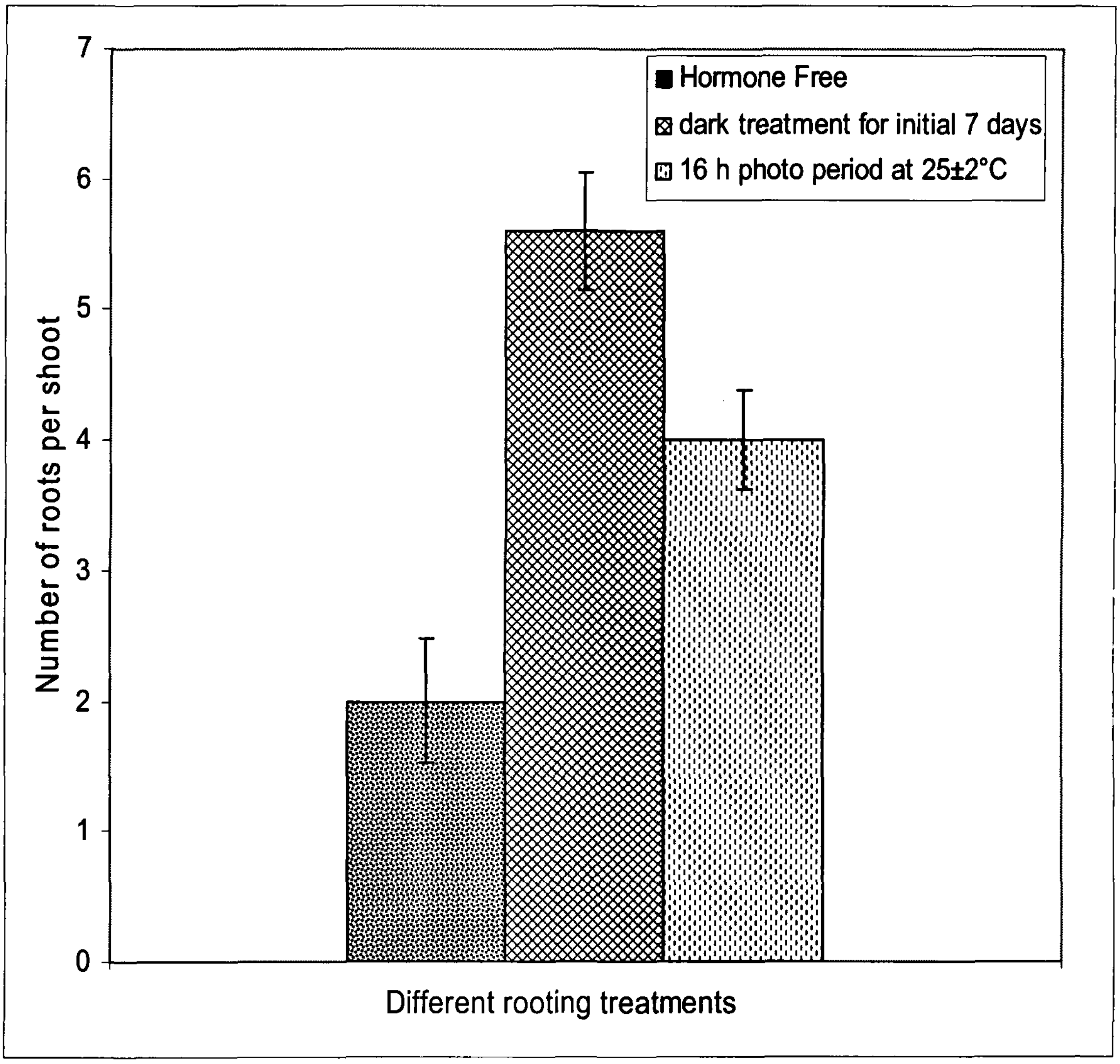


Fig. 3.7. Number of roots developed in ½ MS basal medium after different rooting treatments. Data recorded after 3 weeks

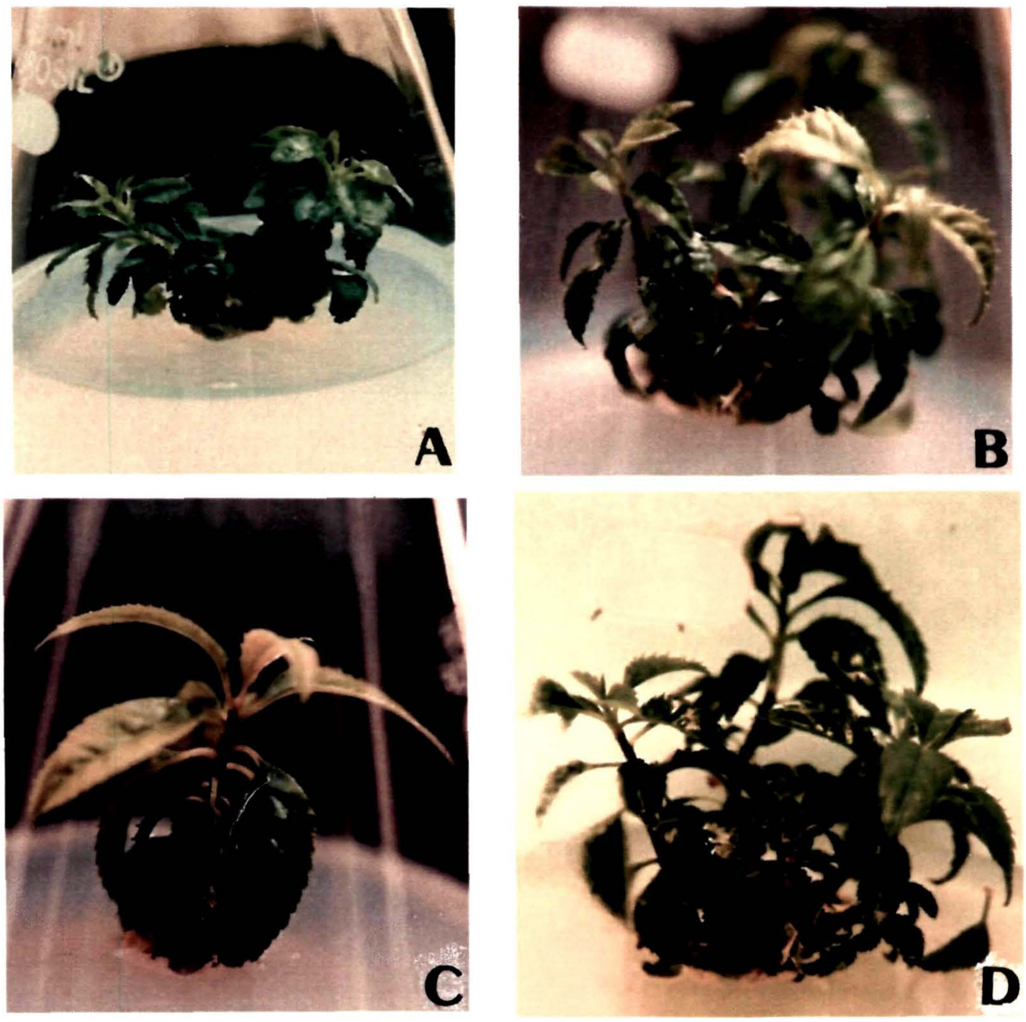
Photoplate 3.1

A - D: Multiple shoot bud induction from nodal stem segments obtained from mature tree in *I. khasiana*

A & B: Direct shoot regeneration in MS + BAP (2.0 mg/l) + KIN (1.0 mg/l)

C: Elongated healthy *in vitro* shoot

D: Shoot with necrotic leaves


A
B
C
D

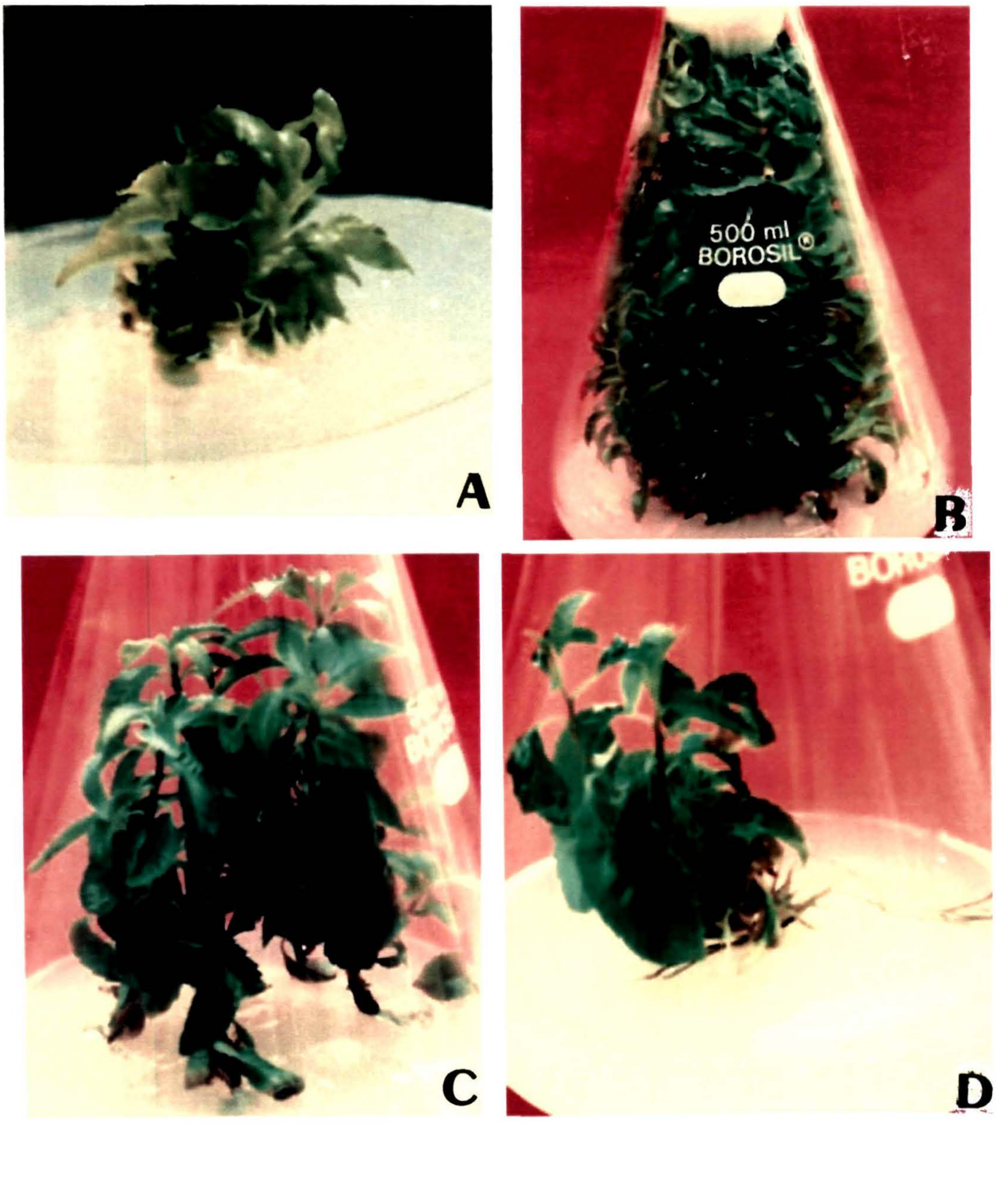
Photoplate 3.2

A - D: Multiple shoot bud induction from nodal segments obtained from young plants of *I. khasiana*

A & B: Direct multiple shoot bud induction on nodal segments on BAP (2.0 mg/l) +KIN (1.0 mg/l)

C: Elongated healthy *in vitro* shoots

D: Shoot with roots after 6 weeks


A
500 ml
BOROSIL®
B
C
D

Photplate 3.3

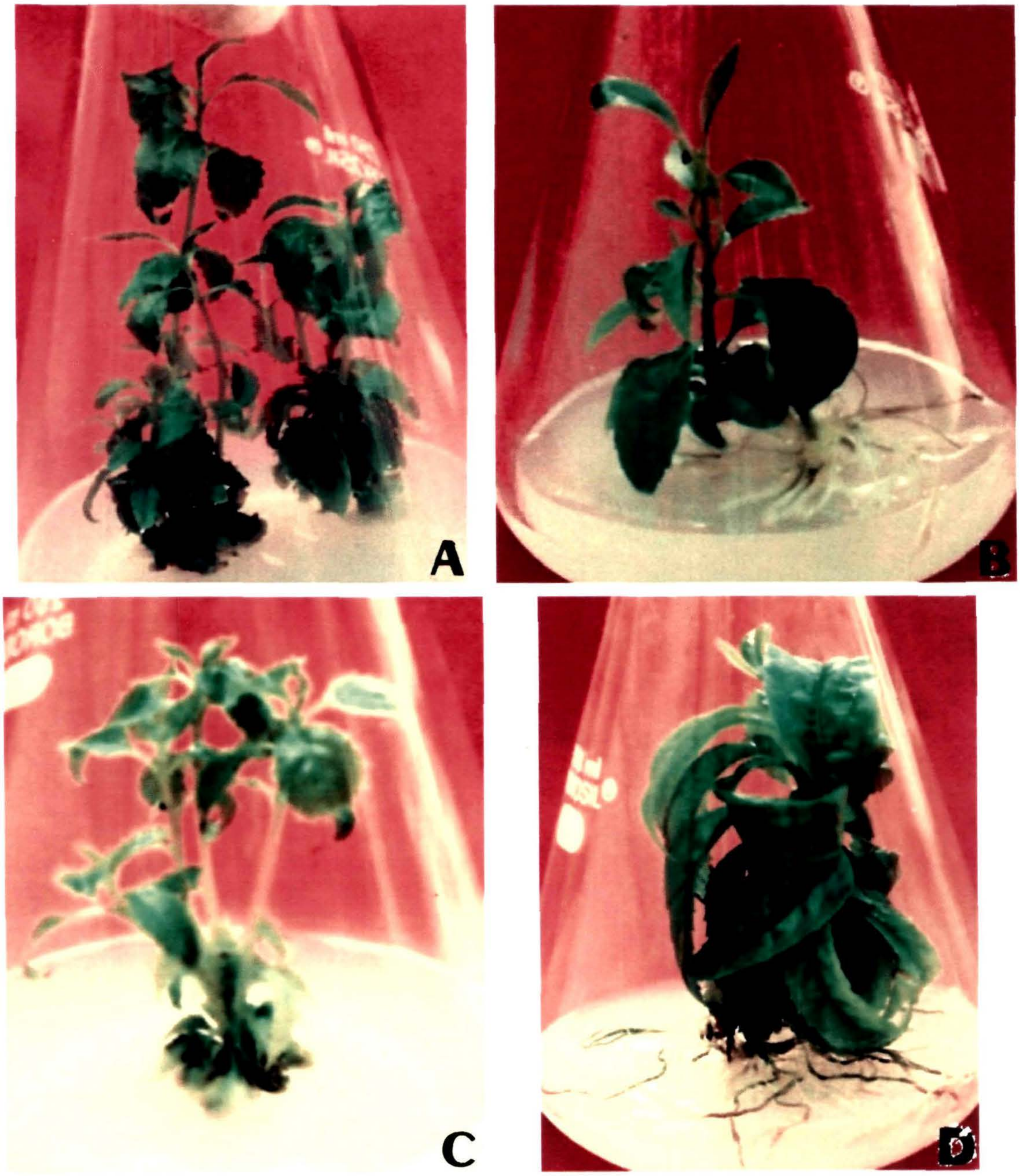
A - D: Shoot development of *I. khasiana* on MS basal salts

A: *in vitro* obtained shoot on MS+ BAP 1.0 mg/l)+ IBA (0.5 mg/l)

B: An elongated shoot producing roots (45 days old)

C: Shoots elongation when explant placed vertically on MS + 1.0 mg/l BAP 0.5 mg/l KIN

D: Root formation on medium with IBA (2.0 mg/l)

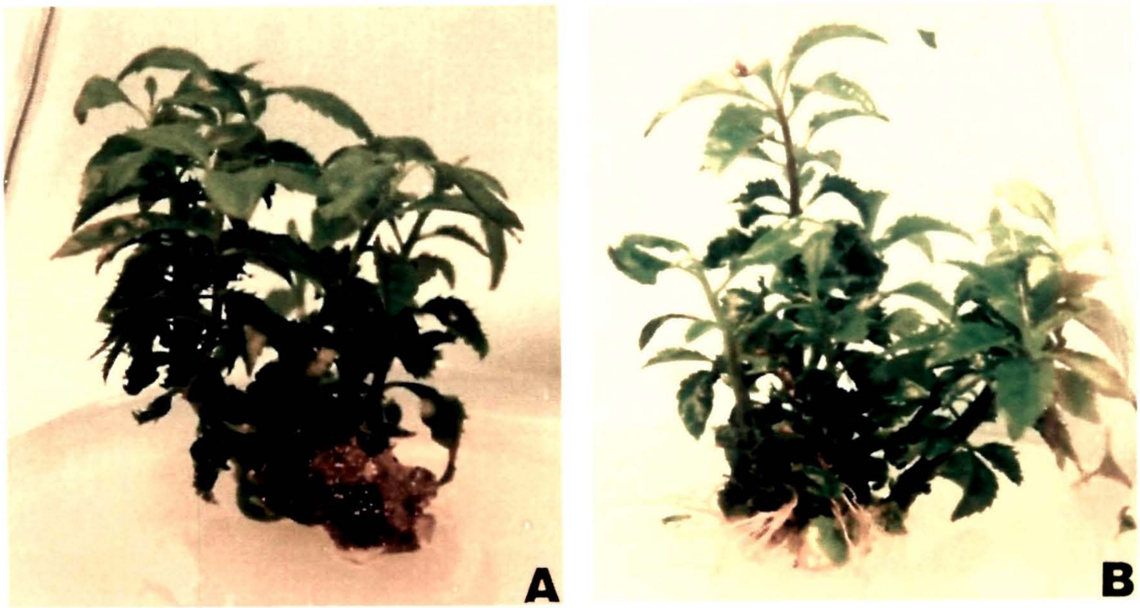

A
B
C
D

Photoplate 3.4

A & B: Multiple shoot in *I. khasiana* on MS medium

A: Multiple shoots with basal callus

B: Multiple shoots with roots (1.0 mg/l) (50 Days)



Photoplate 3.5

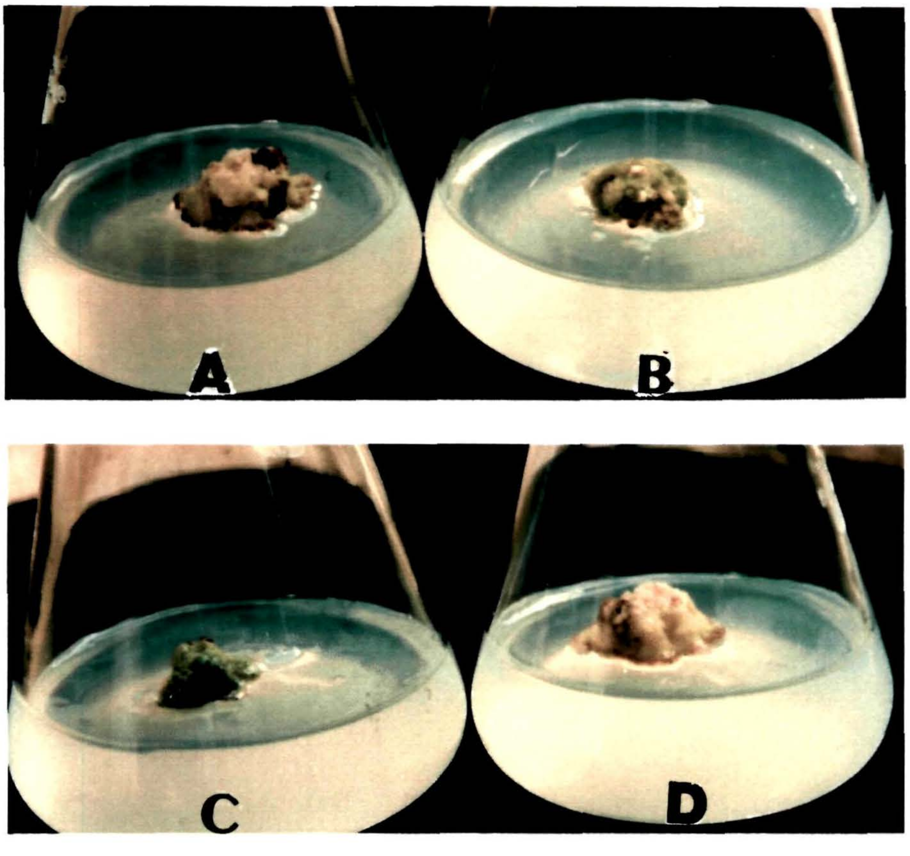
A - D: Callus induction from leaf disc explants on MS + 2,4-D (2.0 mg/l) + KIN 90.5 mg/l)

A: Callus with light brown colour with dark areas at 150 μ moles m^{-2} Sec^{-1}

B: Profuse healthy callus at 50 μ moles m^{-2} Sec^{-1}

C: Slight greenish callus at100 μ moles m^{-2} Sec^{-1}

D: Callus formation with yellow colour at 200 μ moles m^{-2} Sec^{-1}

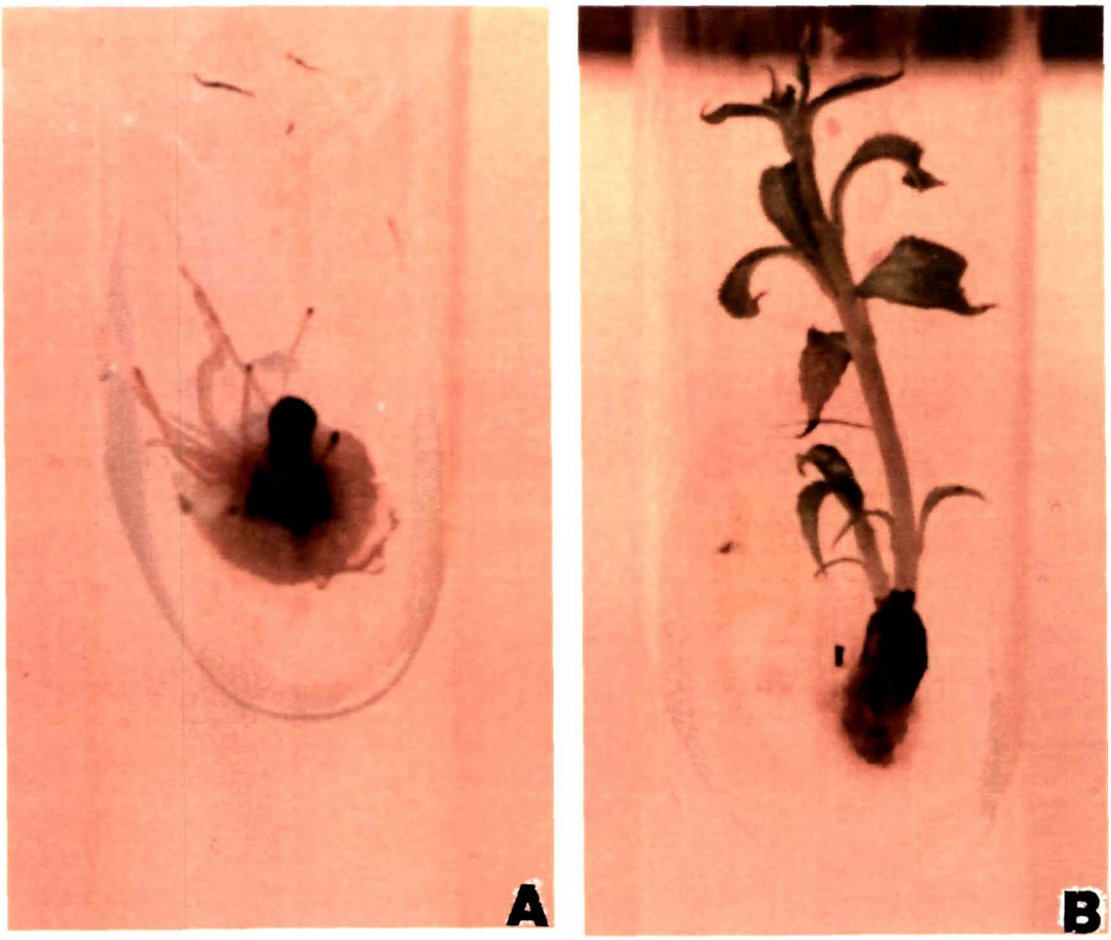

A
B
C
D

Photoplate 3.6

A - B: Callus Induction and organogenesis from leaf discs in *I. khasiana* on MS medium

A: Rooting from nodal explants with callus formation on the basal region on 2.5 mg/l 2, 4-D + 0.5 mg/l KIN

B: Shoot regeneration from nodal explant on 2.5 mg/l KIN + 0.5 mg/l IBA


A
B

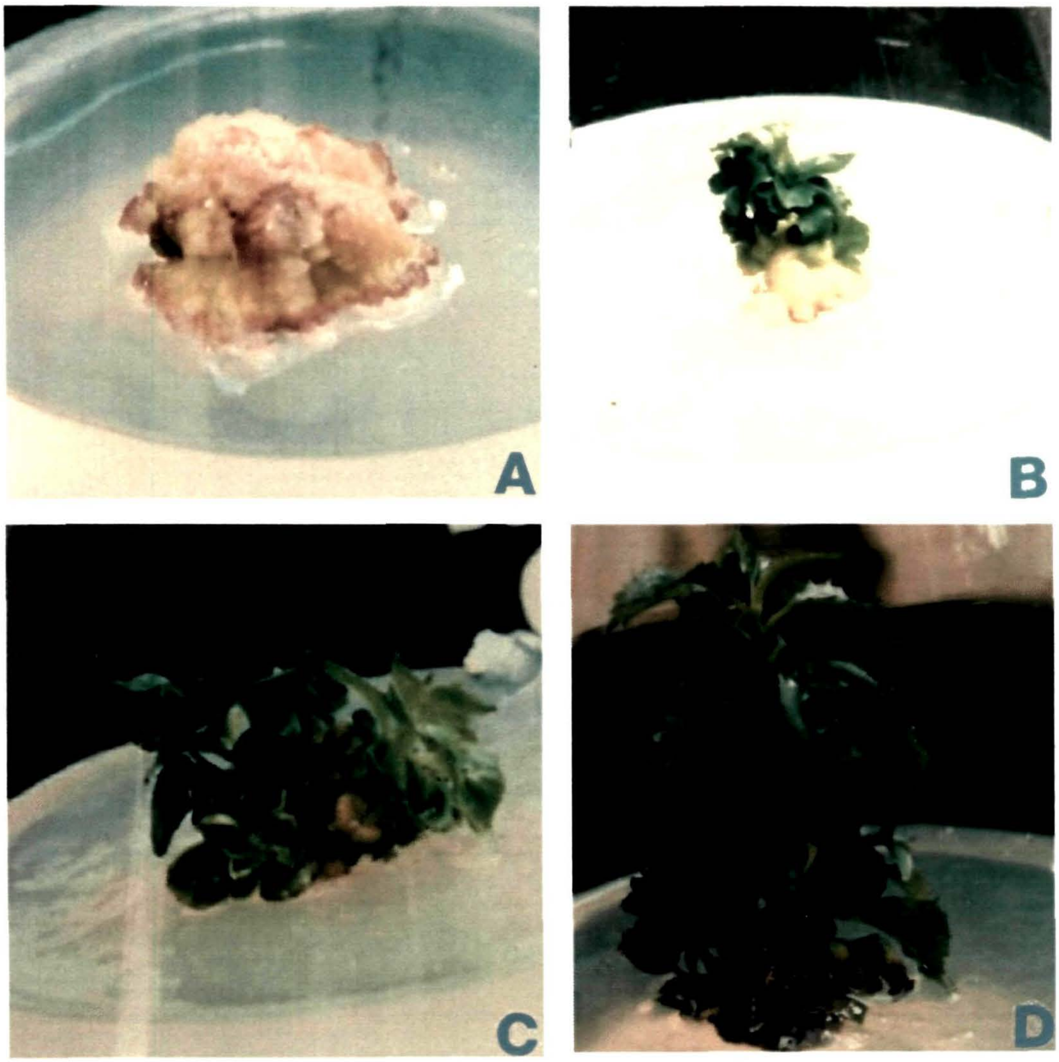
Photoplate 3.7

A - D: Callus induction and organgenesis in *I. khasiana*;

A: Callus formation from leaf disc explants on MS + 2,4- D (2.0 mg/l) + KIN (0.5 mg/l)

B & C: Shoot regeneration from callus on MS + BAP or KIN (2.5 mg/l)

D: Callus formation on the basal region of *in vitro* obtained shoots on MS + BAP (2.5 mg/l)


A
B
C
D

Photoplate 3.8

A - C: Isolated shoot rooted on ½ MS medium + IBA (2.0 mg/l)

A & B: Isolated shoot rooted on medium with IBA (2.0 mg/l) in the dark with in 12 days and shoot rooted in the light after 45 days

C: Shoot branching at basal region with profuse rooting later (60 days old)

cm.
11
10
9
8
7
6
5
4
3
2
1
A
B
C

CHAPTER IV: MICROPROPAGATION OF *NYMPHAEA TETRAGONA*

Introduction

Nymphaea tetragona is a highly endangered aquatic herb confined to a single Nongkrem Pond, Shillong, Meghalaya. This plant species is on the verge of extinction with merely 30-50 plants remaining in the pond surrounded by anthropogenic activity.

A serious obstacle faced by tissue culturists from using hydrophytes as experimental materials is the inherent difficulty in establishing aseptic cultures from plants growing in natural surroundings. The difficulties associated with sterilization of aquatic plants have been attributed to the presence of large bacterial and fungal epiphytic populations, mucilaginous coatings, endophytic organisms in highly lacunate tissues and lack of cutinized epidermis (Madsen, 1985; Godmaire and Nadewajko, 1986).

Keeping in view the highly restricted population of *N. tetragona* and to minimize the problem of surface sterilization and establishment of axenic cultures, the immature and mature but undehisced fruits were taken as starting explants for this present investigation. The presence of hard fruit wall or seed coat protected the embryo from the adverse effects of the sterilizing agents.

Materials and Methods

Seasons vs. Explant Source

The influence of seasons on explant plays an important role in the initiation and establishment of cultures in the media. So selection of suitable source of explants in appropriate season is a vital process for the success of *in vitro* clonal propagation. Various explants from *N. tetragona* aquatic herb were collected from Smit pond, Shillong, Megahlaya. Explants like leaves, rhizome buds, flower buds, pre-mature- or mature undehisced fruits were excised from the plants in four different seasons, Spring (April), Summer (July), Autumn (October) and Winter (December- January)

Surface Sterilization

Premature- or mature undehisced fruits, rhizome tips, petioles, leaf, flower buds were collected from the pond. These explants were washed first with 2% (v/v) teepol for 10 min and then with tap water, followed by 15% (v/v) sodium hypochlorite (NaOCl) solution for 10 min and rinsed 5-6 times with autoclaved distilled water. The explants were further treated with 0.01-0.15 % $HgCl_2$ for 10 min and rinsed thrice with double distilled water to remove all traces of the sterilants. These were then treated with 70% ethanol for 10-30 seconds.

Media Compositions and Conditions

Different media such as MS, ½ MS, LS, Nitsch, White's media were used. Out of the various media tested in the initial experiments, MS medium was found to be the most suitable and it was finally selected. Both liquid and semi solid media were used for initiating *N. tetragona* cultures.

Raising and Maintenance of Aseptic Cultures

i) Direct Multiple Bud Induction

Rhizome explants containing axillary buds were excised from the plant and sterilization was carried out using various surface sterilants like NaOCl, HgCl2, ethanol etc as mentioned in the material and methods.. Thereafter the trimming was done with scalpel blade. Explants like petioles, flower buds besides rhizomes were cultured on MS media supplemented with different auxins and cytokinins at a range of concentrations (0.5 -2.5 mg/l) for direct shoot multiplication. Data were recorded after 4 weeks of cultures.

The percentage of explants producing shoots, number of multiple shoots per explant and shoot lengths were recorded after 4 weeks of culture.

The effects of different photoperiods, dark treatment, GA_3 and different carbohydrates were also studied for optimizing growth and development of rhizome bud cultures.

ii) Root Initiation

For root initiation, isolated shoots were transferred to hormone-free medium or media containing various auxins (IBA, IAA, NAA) in the range of concentrations (0.5-3.5). Observations were recorded after 4 weeks of culture. The number of roots and their lengths were recorded.

iii) Embryo Culture

The pre-mature and mature embryos were isolated from the fruits and cultured on both liquid and semi-solid media containing different growth hormones, cytokinins (BAP, KIN) and auxins (IBA, NAA, IAA) or GA_3 at a range of concentrations (0.5 -2.5 mg/l). These were incubated at 25±2°C temperature and 16 h photoperiod of 200μ mol m^{-2} sec^{-1} intensity. The number of shoots and their lengths were recorded. The effects of different carbohydrate treatments like sucrose (0.5, 3%), galactose (1.0, 1.5%), sorbitol (1-2%), mannitol (1-2%) were also studied in MS medium containing BAP (2.0 mg/ l) in combination with KIN (1.5 mg/l) and NAA (0.5 mg/l) for optimizing the performance of embryo cultures.

The percentage response of embryos showing shoot bud induction, number of shoots and shoot lengths were recorded after 4 weeks of culture. Data were analyzed statistically.

Results

The rate of shoot proliferation was greatly influenced by various seasons during which the explants had been collected. Explants collected in every month showed that shoot proliferation was greatly influenced by various seasons (Table 4.1). Serious contamination was experienced when the explants were derived from the plants during December to February. High rate of opening of axillary buds coupled with maximum number of shoots with less contamination was recorded during the months April to July. The sprouting from the explants in the months of September to November was greatly reduced.

For the establishment of cultures, the surface sterilization treatment with 0.1% $HgCl_2$ for 10 min (Fig. 4.1) and 70% ethanol for 15 secs was found to be the most suitable for undehisced fruits (Fig. 4.2). For rhizome buds, 0.15 % $HgCl_2$ for 10 mins followed by 70 % ethanol treatment for 20 secs resulted in optimal survival rate (Figs. 4.1 and 4.2). The combination of the surface sterilants comprising teepol, mercuric chloride and 70% ethanol resulted in the maximum survival of the explants namely undehisced and rhizome buds (Fig. 4.3).

Rhizome Bud Culture

Out of the various media tried namely MS, Nitsch and White along with various concentrations of BAP, it was found that the maximum number of shoots per rhizome bud could be accomplished on MS medium containing 2.0 mg/l BAP (Fig. 4.4). The rhizome buds cultured on Nitsch and White media containing the same concentration of BAP resulted in fewer shoot buds per explant. Further reducing the strength of basal salts of the same MS medium to half and one fourth, it was found that full strength MS medium resulted in maximum response of the explants (Table 4.2).

Effect of BAP + Auxins

For the establishment of direct shoot induction, MS basal medium supplemented with different concentrations of BAP (0.5-2.5 mg/l) along with IAA (0.5 mg/l) showed varying results (Table 4.3). The results showed that BAP at 2.0 mg/l with 0.5mg/l IAA in medium was the most effective cytokinin for inducing shoot formation in *N. tetragona* wherein a maximum number of 3-4 shoots per explant were produced. The decrease or increase of the concentration of BAP in the medium did not enhance the number of shoots (Table 4.3).

Effect of Cytokinins

Of the two cytokinins (BAP, KIN) tried, BAP was more effective than KIN in inducing multiple shoots from rhizome tip explants. Shoot formation was reduced to 1-2 in number in KIN supplemented medium. The combination of both the cytokinins (BAP + KIN) at different concentrations did not enhance bud multiplication from rhizome explants of *N. tetragona.*

Elongation of *In Vitro* Shoots

For shoot multiplication and elongation, shoots derived from rhizome cultures were subcultured on MS medium containing BAP (2.0 mg/l) + IAA (0.5 mg/l). The shoots attained a height of 3-4 cm length in 3 weeks bearing well developed healthy leaves with stout petioles (Photoplate 4.1 A,B).

Effect of Photoperiods

Subjecting the cultures to different photoperiods, it was found that out of all the photoperiods tried, 16 h light period induced the maximum number of shoots having optimal length on MS medium containing 2.0 mg/l BAP and 0.5 mg/l IAA after 4 weeks time (Table 4.4).

Effects of Carbohydrates

The optimal shoot regeneration was recorded on rhizome buds cultured on the same MS medium containing 3% sucrose. Decreasing the concentration of sucrose in the medium resulted in decline of shoot regeneration. Incorporation of other carbohydrates like sorbitol, galactose and mannitol could not improve the regeneration of the shoots (Fig.4.5). Infact, galactose in the medium was inhibitory for the regenerating shoots of *N. tetragona.*

Rooting of Shoots

The elongating shoots were transferred to growth regulators free-medium or reduced concentrations of IBA (2.0 mg/l) alone. The shoots rooted in both the media within 4 weeks. But an average 3 roots per shoot was observed in hormone-free medium after 20-30 days of culture (Photoplate 4.2 A).

Embryo Culture

The optimal shoot number was observed from pre-mature embryos cultured in MS medium containing 2.0 mg/l BAP and 0.5 mg/l NAA wherein a maximum of 12 shoots per explant were found to have emerged (Table 4.5). In the same concentration the shoot length was also seen to be slightly enhanced. However, addition of GA_3 at 0.5 -2.5 mg/l in the medium enhanced the shoot number slightly whereas the length of the shoots was markedly enhance (Photoplate 4.3 B).

Effect of Cytokinins

Incorporation of various cytokinins namely BAP, KIN and zeatin singly in the range of concentrations 1.0- 2.5 mg/l in the medium along with 0.5 mg/l NAA brought varying response of the embryos (Fig. 4.6). It was seen that out of all the cytokinins tried, BAP at 2.0 mg/l in the medium resulted in around 12 number of shoots per embryo. Zeatin in the medium was found to be inhibitory for the induction of shoot buds from the embryo.

Healthy plantlets of *N. tetragona* could be regenerated on both liquid and semi-solid medium supplemented with 2.0mg/l BAP and 0.5mg/l NAA (Photoplate 4.4 A,B).

Out of various carbohydrates used, sorbitol (1%) in the medium was found to be the best for the regeneration of the embryo and was followed by sucrose(3%). Incorporation of galactose or mannitol were found to be inhibitory for the induction of shoot buds from the embryo (Fig. 4.7).

Out of the different explants (premature embryo, rhizome bud, petiole and flower bud), premature embryo and rhizome buds were found to be the best sources of explant for the induction of shoots. Petiole and flower bud showed less response (Fig. 4.8).

Discussion

The selection of explants is the most important factor that should give maximum number of shoots or morphogenetic response within a short time. The rhizome buds, petioles, flower buds, immature or mature undehisced fruits, leaf discs etc. have been tried as explants. In the present study, rhizome buds and undehisced immature or mature fruits have been shown to be the most suitable explants. The maximum response of the rhizome buds reported in the present study might be due to the presence of apical and cambial meristem inside the explant tissues, as opined by various workers (Murashige (1974), Ammirato (1983), Bajaj (1986) and Gamborg and Phillips (1986)).

Age of the explants play a major role in the tissue culture experiments (Ammirato, 1983; Gamborg and Phillips, 1986). In our present study, 3-4 months old rhizome was found to be better. This high response rate of the explants may be due to the increased physiological activity of the rhizome, which is being transferred to the growth regions like buds and meristems.

The optimum surface-sterilization of *N. tetragona* was obtained using the following steps; washing of explants with teepol (2%) for 10 min followed by 15% (v/v) sodium hypochlorite (NaOCl) solution for 10 min and rinsed 5-6 times with auatoclaved distilled water; treatment with 0.1% $HgCl_2$ solution for immature undehisced fruits for 10 min; 0.15% $HgCl_2$ for rhizome buds for 15 min, treating with 70% alcohol about 15-20 seconds, rinsing with double distilled water (about 3 times). $HgCl_2$ increased the survival rate of explant by resisting the growth of inherent microbial contaminants of rhizome explants. The hard seed coat of immature fruits could also resist the sterilants without destroying the cover of the pre-mature embryo. Therefore, the immature undehisced fruit was the best source of materials to avoid the inherent difficulty of aquatic culture. The inherent difficulties in the establishment of axenic aquatic cultures due to surface disinfestation have been attributed to the presence of a large bacterial and fungal populations, mucilaginous coating, endophytic organism in highly lacunate tissues and lack of cutinized epidermis (Madsen, 1985. Godmaire and Nalewajko, 1986). The suitable selection of the sterilizing agents mainly depend upon the factors like nature of explants collected (underground or not) extend of surface microflora and the sensitivity of the explant tissues to various sterilizing agents, optimum treatment time and concentration of sterilizing agents etc. and should be determined by trial and error methods. However, once contamination- free culture obtained, that could be used for further culture studies. The treatment time vary with different species or nature of explants used for the culture (Nadgauda *et al.,* 1991; Sakamura and Suga, 1989).

Cytokinins induce cell division and activate the dormant buds of the explants to enlarge and grow (Sachs and Thiman, 1964). As reported it was found that BAP played an important role in this study and resulted in the production of 3-4 shoots per explant. The superior effect of BAP has been emphasized in many Zingiberaceous medicinal plants such as *Zingiber* and *Curcuma* (Balachandran *et al.,* 1990) where in total of 3-4 shoot buds in MS medium supplemented with 3.0 mg/l) BAP were obtained. Lakshmanan (1994) also reported the stimulatory effect of BAP in producing 3-4 shoots per rhizome tip culture in *Nymphaea* hybrid "James Brydon' (a hybrid composed of *N. alba, N. candis* and *N. laydekeri*) showed decreased frequency of shoot induction (Table 3). The increased concentration of BAP might have imparted a toxic effect on the explants by retarding cell division. Werner and Boe (1980) had also noticed abnormal growth/dying of cells, when explants cultured in the presence of BAP, above its optimum concentration. Other investigators like Mathew (1993) and Sahoo and Chand (1998) also reported similar arguments of Werner and Boe (1980). Sijina *et al.*(1997) found that rhizome buds showed superior shoot bud induction in *Amomomum subulatum*. Hu and Wang (1983) reported that most of the micropropagation reports (68%) amplify the superior effect of BAP, with comparatively very low percentage (22%) to the promotive effect of KIN on micropropagation.

Direct multiple shoot regeneration aims at the production of disease –free plants, true-to-type progeny, on large scale in limited time. A number of plant species has been worked out using rhizome buds/shoot tips as source of explants. viz., *Limnophila indica* (Roa and Mohan Ram, 1981); *Nelumbo lutea* (Kane *et al.*, 1988); *Curcuma amada* (Barthakur and Bordoloi, 1992) and *Zingiber officinale* (Nirmal Babu *et al.*, 1996); Sharma and Singh, 1997).

In the present study, incorporation of lower concentration of IAA at 0.5 mg/l along with cytokinin BAP induced more number of shoots, than without auxin in the medium. The possible roles of auxin in the shoot multiplication stage is to nullify the suppressive effect of high cytokinin in the medium, when the two interact together. Higher concentration of auxin may induce callus from explants. The balance between auxin and cytokinin regulate the growth and differentiation (Debergh and Zimmerman, 1991; George, 1993a). Many reports are available, in which auxin cytokinin ratio favoured shoot multiplication. For example, Barthakur and Bordoloi (1992) reported that rhizome buds of *Curcuma amada* produced 7-12 shoots and roots simultaneously on MS medium supplemented with 0.5 mg/l NAA and 4.0 mg/l BAP. Similar result were obtained by Samarajeewa *et al.,* (1993) where adventitious shoot induction was reported in *Gloriosa superba* on MS medium containing 0.05 mg/l BAP + 0.01 – 0.05 mg/l, IBA, IAA or NAA. Lakshmanan (1994) also reported higher number of shoot bud proliferation in rhizome tips of *N. hybrid* "James Brydon' on MS medium containing 8.0 mg/l NAA, 32.0 mg/l 2 iP and 11.1 mg/l BAP in concentration. Synergestic effects of other auxins and BAP on promoting shoot multiplication of medicinal plant species have been well documented, e.g. BAP + 1BA in *Piper nigrum* (Phillip *et al.,* 1992) and BAP + NAA in *Gomphrena officinalis* (Mercier *et al.,* 1992). However, Kane *et al.*(1988) reported neither BAP nor Zeatin had a significant effect on *in vitro* growth and development of *Nelumbo lutea* rhizome bud. Jenks *et al.* (2000) reported BAP was most effective in stimulating adventitious shoot formation in combination with IAA on MS basal medium. Shoot formation was completely inhibited in the presence of BAP alone in *Nymphoides indica* (Jenks *et al.*, 2000). Similar results of ineffectiveness of BAP alone were reported in internodal stem explants of *Myriophyllum aquaticum* (Kane *et al.*,1991).

Out of various media tried, MS medium was found to be better than the others for induction of shoot buds. Major reduction in the level of nutrients in Nitsch or White media as compared to MS might have adversely affected the multiple shoot induction. MS medium supplemented with 2.0mg/l BAP resulted in the maximum number of shoots per explant, whereas the same concentration of BAP in other two media (Nitsch and White) did not induce multiple shoot bud induction. Agretious *et al.* (1996) also reported that White medium was less effective in *Alpinia calcarata*.

The photoperiod seems to influence the rate of multiplication of shoot buds and their length. The 16 h photoperiod was found to be suitable to influence shoot number and length, with three to four shoots of 4-5 cm length as compared to cultures incubated in dark (with one shoot of 2-3 cm). Lakshmanan (1994) also reported 16-h photoperiod to be stimulatory for increased shoot length and multiplication about 1.5 and 2.4 times respectively, over dark incubated cultures. Similar report of partial photoperiodic regulation on the growth of *N. 'gladstone'* was observed by Kelly and Frett (1986).

Of all the carbohydrates (sucrose, galactose, sorbitol, mannitol), sucrose at 3% was found to be the best on shoot regeneration percentage of rhizome bud. More than 70% shoot regeneration was recorded. Increasing or decreasing the sucrose concentration from 3% inhibited the percentage of shoot bud sprouting, and other carbohydrates like galactose, sorbitol and mannitol in the medium were not effective. The carbohydrate source has been shown to be an important factor for *in vitro* growth and development in affecting both somatic embryogenesis and embryo maturation (Scott and Lyne 1994; Nuutila *et al.*, 2000).

The regeneration percentage of cultured embryo of *N. tetragona* was quite low on MS medium. The combination of 2.0 mg/l BAP and 0.5 mg/l NAA resulted in 12 shoots per embryo. However, the addition of GA3 in the same medium enhanced the shoot length from 4.4 cm to 9.2 cm. The removal of either BAP, NAA or GA3 did not give satisfactory result. Jenks *et al.* (2000) reported maximum shoot regeneration (11.5 shoots per explant with 80% responsive explants) occurred on explants cultured on basal medium supplemented with both 10µM BAP and 20µM IAA. They also found that shoot organogenesis was completely inhibited in the presence of 10µM BAP alone. Priyadarshan *et al.* (1988) succeeded in the clonal propagation of cardamom in medium, fortified with IAA and BAP..However, Kane *et al.* (1988) showed that neither BAP nor Zeatin had a significant effect on *in vitro* growth and development of *Nelumbo ltutea* rhizome explants, another Nymphaceae member. But, they also found that GA_3 significantly promoted rhizome growth in *N. lutea.*

The addition of sorbitol along with 2% sucrose was found to be the best for embryo regeneration with the increase in response from 65-95%. This work was carried out on the basis of earlier research performed by Li *et al.* (2004). They found that the combination of 1% sorbitol and 0.5% sucrose in the medium increased the percentage frond regeneration from calli of *Spirodella puntata.* Frick (1991) reported that *Lemna* minor plants efficiently assimilate glucose, fructose, mannitol and starch, but their growth is inhibited by galactose. Many such results exist that callus growth and morphogenesis can be prominently influenced by other carbohydrate supplements (Swedlund and Locy, 1993; Jeanin *et al.*, 1995; Jain *et al.* 1997; Lemos and Baker, 1998; Li *et al.*, 2004).

The purpose of this study was to develop a mass *in vitro* propagation system for *N. tetragona* as its population is extremely low with only 30-40 plants existing in a single pond. Micropropagation may be useful to increase the production of disease free clonal material. Tissue culture also provides opportunity to improve traditional ornamental plants by inducing random genetic variations through mutation breeding for germplasm preservation and ornamental purpose. (Broertjes and Leffring, 1972; Broertjes and Van Harten, 1978; Ibrahim *et al.*, 1998).

Table 4.1. Influence of the season on the percentage response of rhizome bud of *N. tetragona* in MS basal medium

Months of collection	**(%) explant response**
February - March	20
April - May	60
June - July	50
August - September	30
October - November	5
December - January	-

Table 4.2. Effect of different strengths of MS medium supplemented with BAP (2.0 mg/l) + NAA (0.5 mg/l) on shoot multiplication from rhizome bud of *N. tetragona*

Basal Media	**Percentage Response**	**Average No. of Shoots (±S.D.)**
MS full	80	3.8 ± 0.68
MS half strength salts	60	2.6 ± 0.83
MS ¼th strength salts	50	1.8 ± 0.5

± SD – Standard deviation

Data represents an average of 10 explants per treatment

Table 4.3.Organogenesis from rhizome explants of *N. tetragona* on MS medium supplemented with BAP and IAA.

Growth Regulators (mg/l)		**(%) Explant response**	**Average no. of shoots**	**Average shoot length (cm)**
BAP	IAA			
0.0	0.5	20	1.0 ± 0.23	1.2 ± 0.32
0.5	0.5	30	1.3 ± 0.28	2.0 ± 0.48
1.0	0.5	50	1.5 ± 0.46	2.8 ± 0.34
1.5	0.5	60	2.7 ± 0.24	3.9 ± 0.40
2.0	0.5	80	3.8 ± 0.58	4.2 ± 0.38
2.5	0.5	70	2.8 ± 0.34	3.2 ± 0.47

± SD

Each cut rhizome explant contains single bud.

Each concentration consisted 10 replicates.

Each regenerated shoot bears its leaf.

Data recorded after 4 weeks

Table 4.4. The effect of different photoperiods on the shoot number and length of rhizome bud cultured in MS + BAP (2.0 mg/l) + IAA (0.5 mg/l) after 4 weeks of culture.

Treatments	**Mean shoot number (± S.D)**	**Mean shoot length (cm) (± S.D)**
Control	1.0 ± 0.0	2.5 ± 0.2
Photoperiod (h)		
0	1.4 ± 0.43	2.4 ± 0.35
8	2.9 ± 0.25	3.0 ± 0.42
16	3.8 ± 0.38	4.2 ± 0.56
24	2.7 ± 0.56	3.2 ± 0.36

± SD

Control: MS medium without growth regulators in the dark.

Table 4.5. Effect of various combinations and concentrations of BAP, NAA and GA_3 on the organogenesis from premature embryos in *N. tetragona* on MS medium

Growth regulators (mg/l)			Mean shoot number	Mean shoot length (cm)
BAP	NAA	GA_3		
0.0	0.0	-	2.0 ± 0.0	2.0 ± 0.2
0.5	0.5	-	2.0 ± 0.0	2.4 ± 0.2
1.0	0.5	-	6.4 ± 0.0	2.6 ± 0.68
1.5	0.5	-	8.6 ± 0.46	2.8 ± 0.36
2.0	0.5	-	12.0 ± 0.56	4.4 ± 0.46
2.5	0.5	-	8.4 ± 0.46	3.2 ± 0.54
0.0	0.5	-	6.4 ± 0.46	2.2 ± 0.4
2.0	0.5	0.5	11.0 ± 0.5	7.8 ± 1.2
2.0	0.5	1.5	12.0 ± 0.5	9.2 ± 0.5
2.0	0.5	2.5	10.0 ± 0.4	8.0 ± 1.4

± SD

Each mean is based on 20 replicates per treatment

Data recorded after 4 weeks

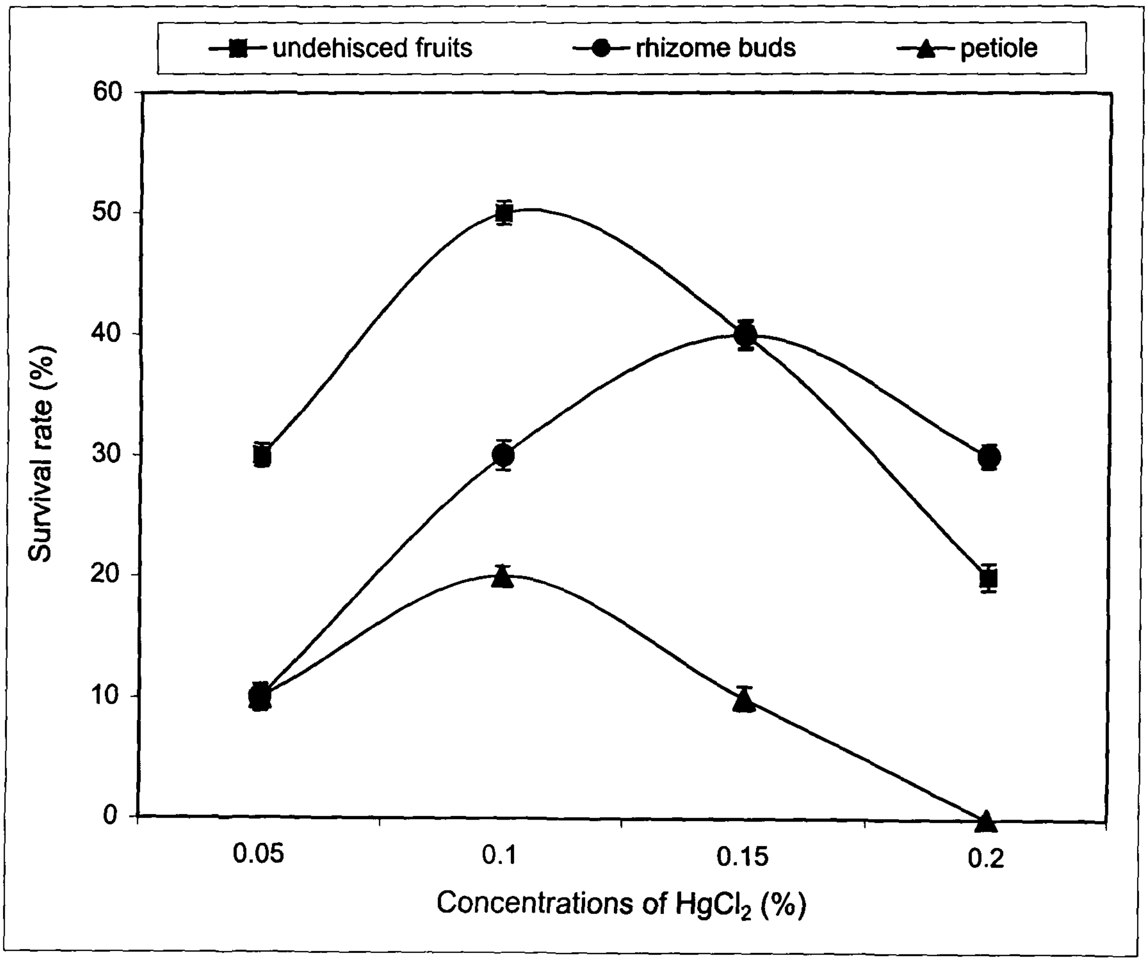


Fig. 4.1. Effect of different concentrations of $HgCl_2$ on the survival rate of cultures of *N. tetragona*

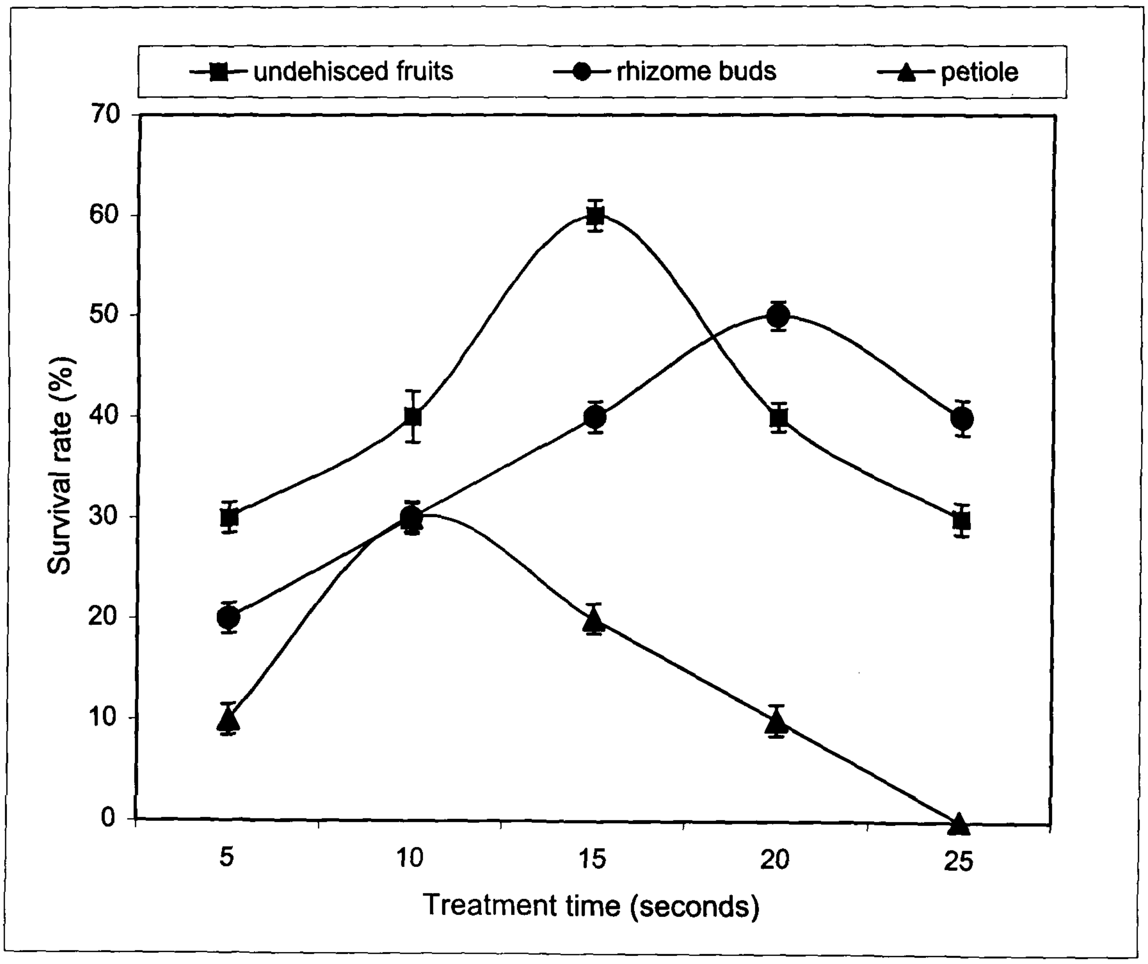


Fig. 4.2. Effect of various time intervals on the survival rate of explants treated with 70% ethanol pretreated with 0.1 % $HgCl_2$ for 10 min

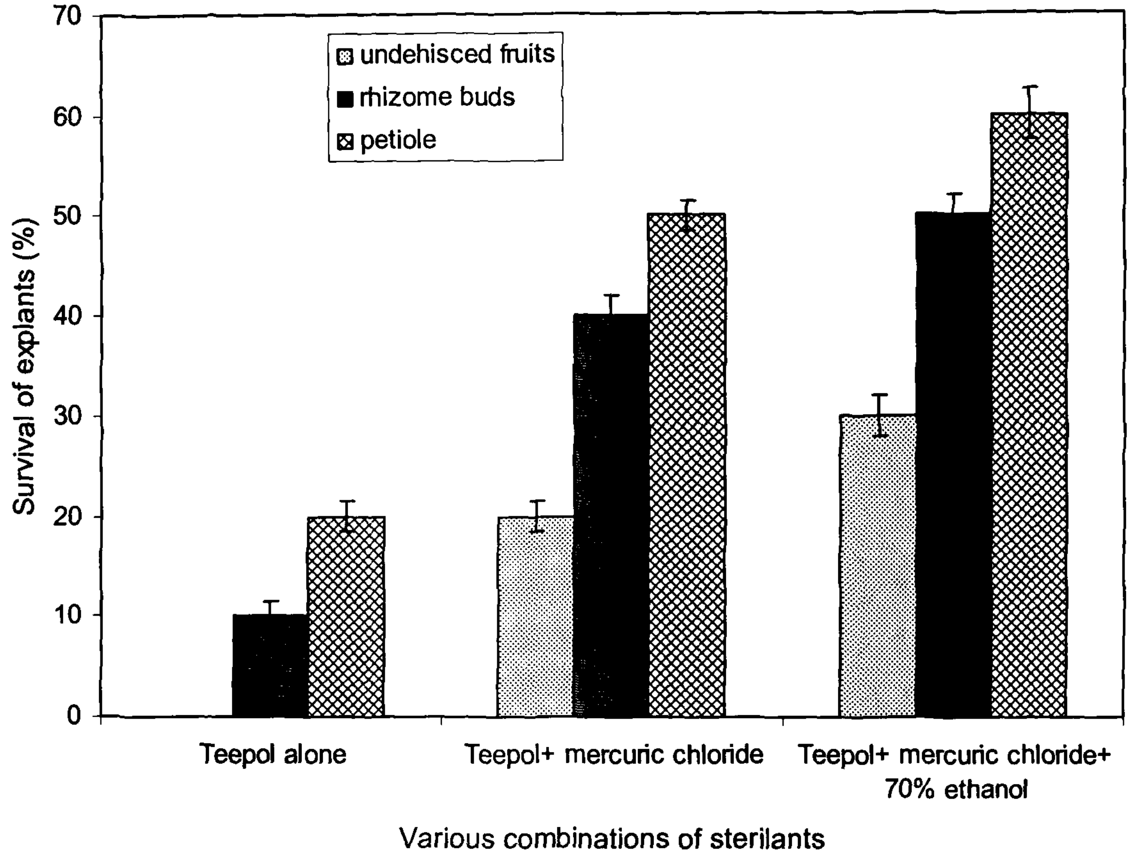


Fig. 4.3. Comparison of the most suitable combination of sterilants on the survival rate of explants

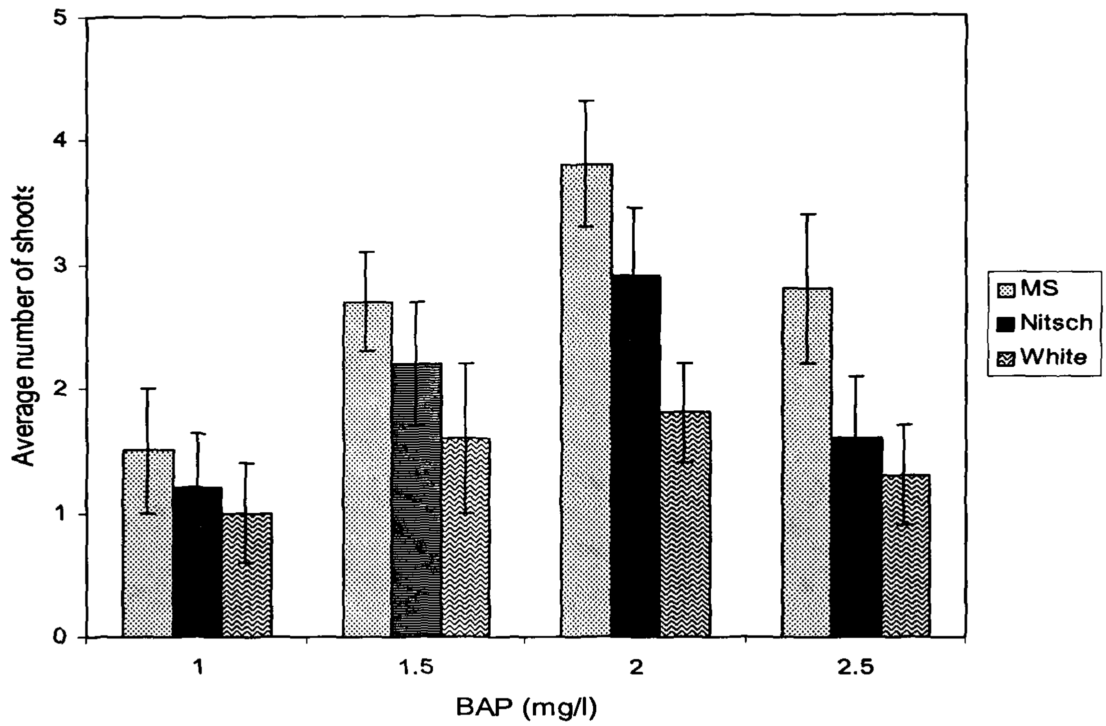


Fig. 4.4. Effect of various media with different concentrations of BAP on the rate of multiplication of shoots from rhizome bud in *N. tetragona*

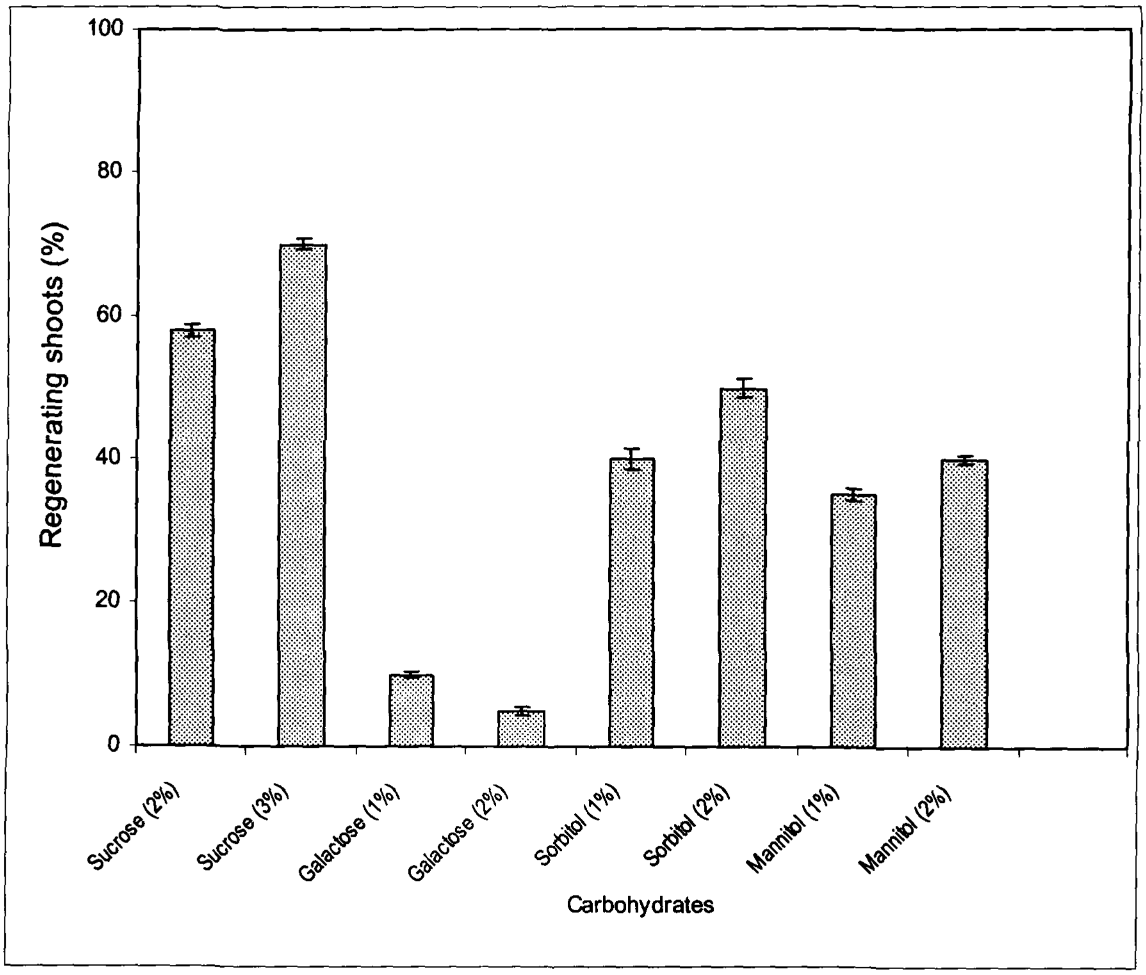


Fig. 4.5. Effect of different carbohydrates on the shoot regeneration percentage of *N. tetragona* rhizome bud

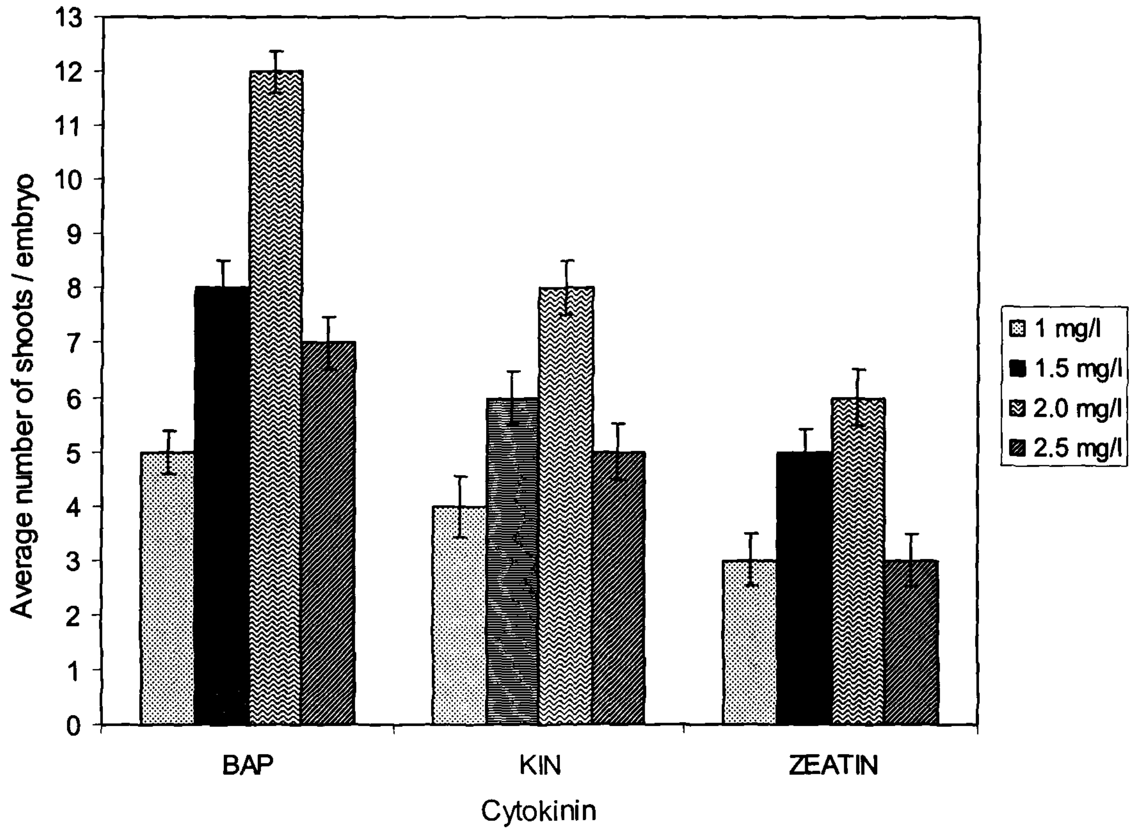


Fig. 4.6. Effect of cytokinins on shoot multiplication of *N. tetragona* embryo in MS + NAA (0.5 mg/l)

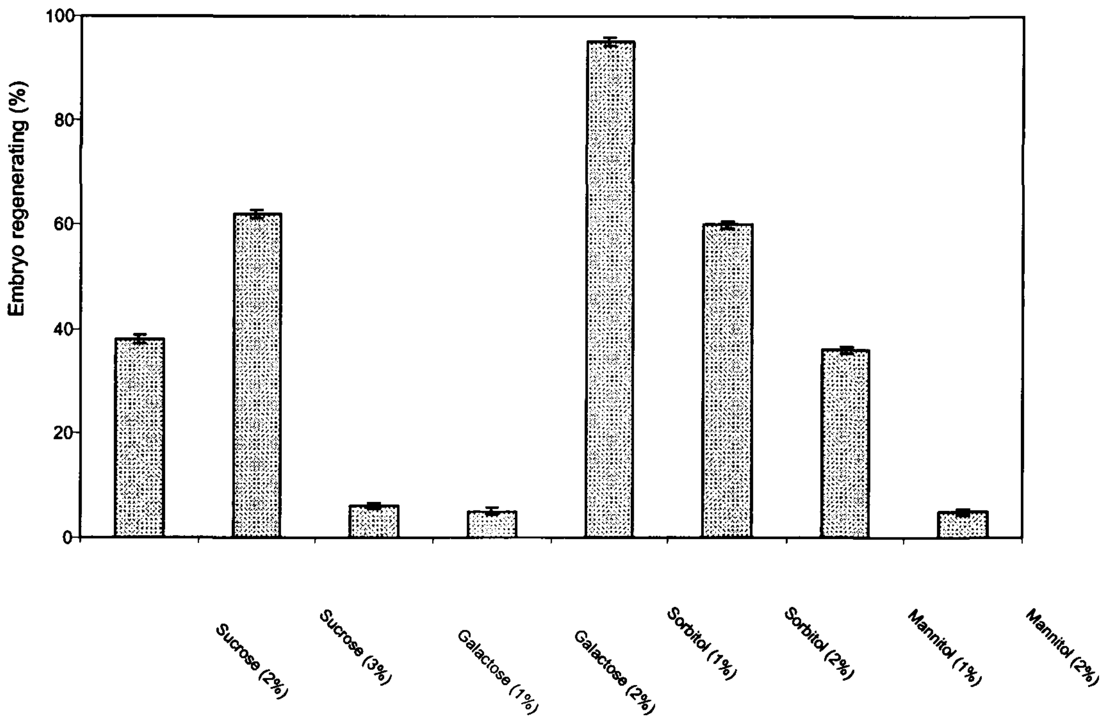


Fig. 4.7. Effect of carbohydrates on the regeneration percentage of *N. tetragona* embryo cultured in MS medium containing BAP (2.0 mg/l) + NAA (0.5 mg/l)

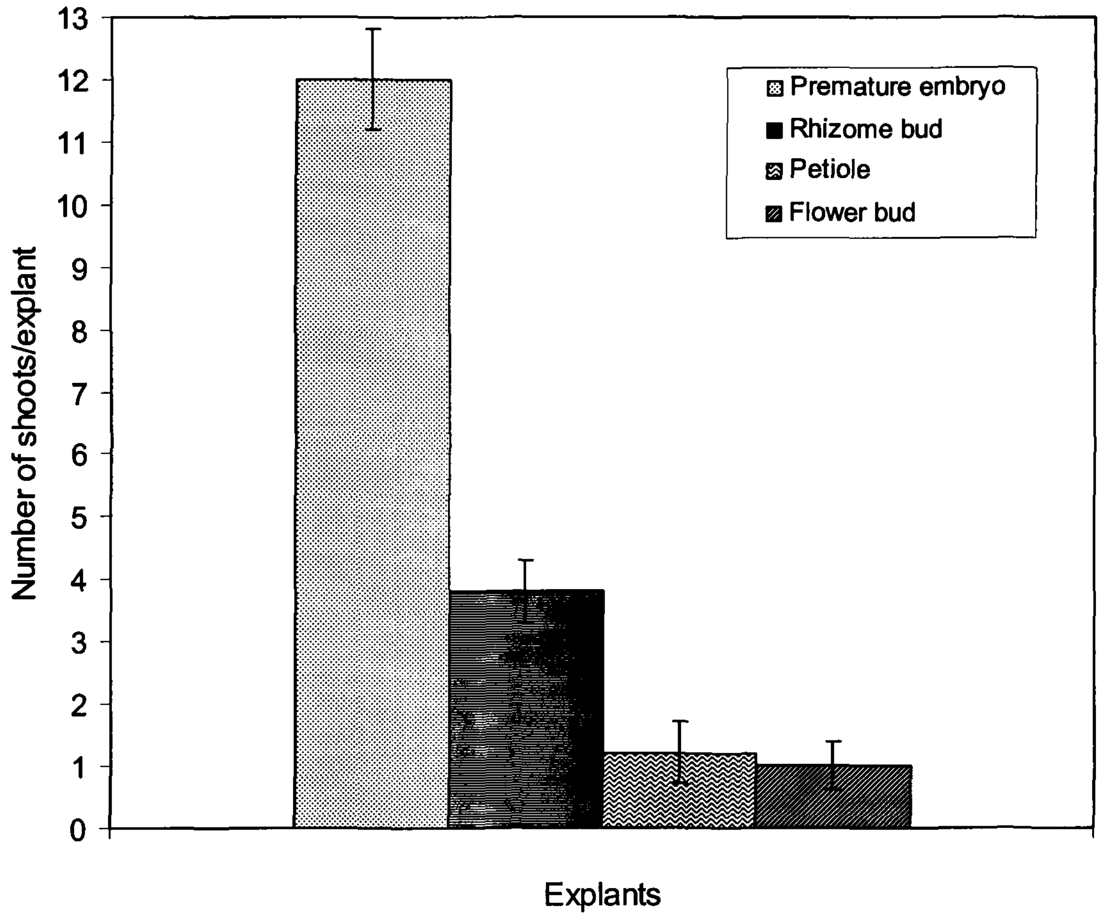


Fig. 4.8. Response of various explants of *N. teragona* in MS + BAP (2.0 mg/l) +NAA (0.5 mg/l)

Photoplate 4.1

A - B: Direct shoot bud induction from rhizome bud

A: Direct shoot regeneration from rhizome bud in MS+ BAP (2.0 mg/l) + IAA (0.5 mg/l)

B: Elongated *in vitro* shoot (4 weeks old)

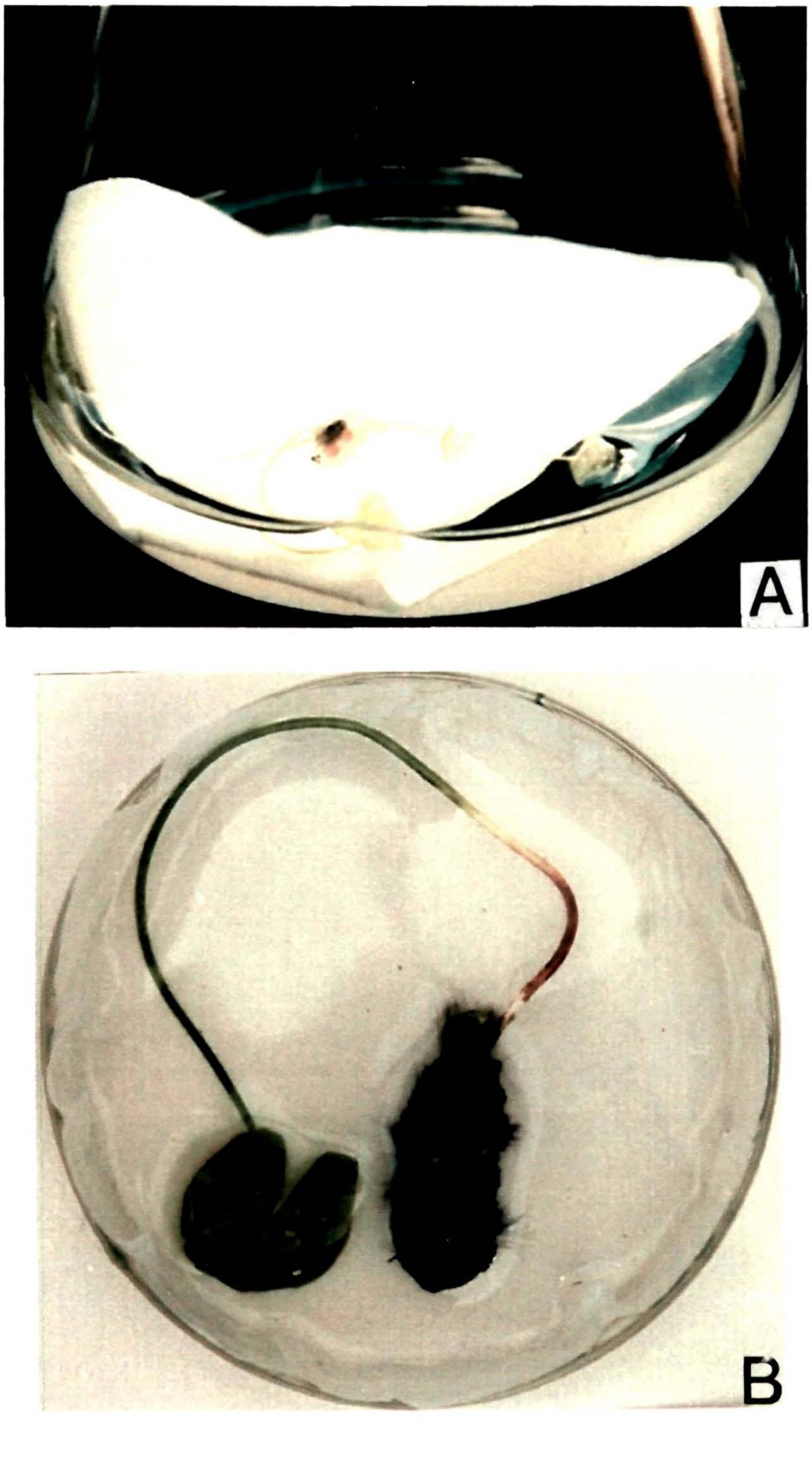

A
B

Photoplate 4.2

A - B: Shoot induction in *N. tetragona*

A: *In vitro* obtained shoot producing roots in MS + IBA (2.0 mg/l)

B: *In vitro* healthy shoot with dark green leaf (16 h photoperiod)

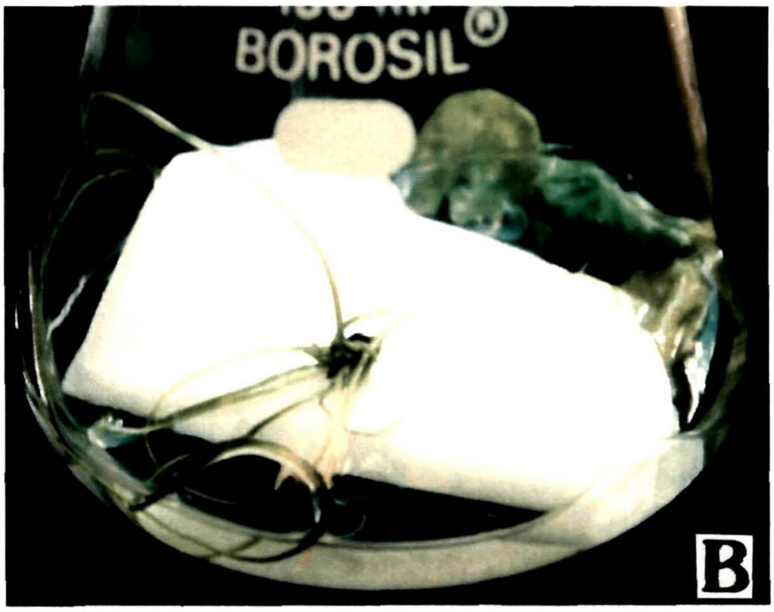

A
B

Photoplate 4.3

A - B: Plantlet regeneration from pre-mature embryos in MS liquid medium + BAP (2.0 mg/l) + NAA (0.5 mg/l)

A: Multiple shoot regeneration at 16 h photoperiod

B: Shoot regeneration with more elongated shoot length in MS liquid medium + BAP (2.0 mg/l) + NAA (0.5 mg/l) + GA_3 (1.5 mg/l)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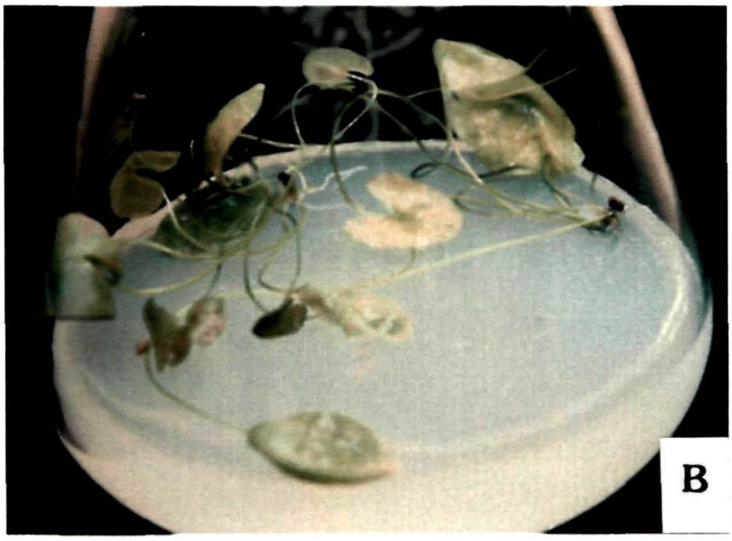
Photoplate 4.4

A - B: Plantlet regeneration from mature embryo in MS medium + BAP (2.0 g/l) + NAA (0.5 mg/l)

A: Multiple shoot induction with healthy leaves on liquid medium at 16 h photoperiod

B: Plantlet regeneration with healthy leaves and roots on semi-solid medium


A


B

CHAPTER V: *IN VITRO PRESERVATION* OF *ILEX KHASIANA* AND *NYMPHAEA TETRAGONA* USING DIFFERENT SLOW GROWTH APPROACHES

Introduction

Threats to biodiversity posed by the destruction of natural habitat notably by ever increasing human population, urbanization, unplanned human activities and global warming has led to an extinction of a large number of plant species (Wilkins, 1991). Many of the plant species have become rare and endangered. So preservation of any such species is required to prevent further extinction.

Conservation aims at maintaining essential ecological processes and life support systems, preserving genetic diversity, and utilizing species and ecosystem sustainably. There are basically two systems for conservation of germplasm- the *in vitro* and conventional means of field / natural conservation i.e., *ex situ* and *in situ* conservation.

In vitro germplasm storage is becoming increasingly vital and forms an important part of *ex-situ* conservation strategy. *In vitro* storage is a necessity for those plant species for which domesticated cultivars do not produce seeds, or species which produce seeds but are highly heterozygous and are usually vegetatively propagated, or those species which produce recalcitrant seeds where the seeds quickly lose viability under conventional seed storage conditions.

The *ex situ* conservation or conventional means of germplasm conservation is by way of maintenance of clonal materials of diverse genotypes under field or glasshouse conditions. For example, vegetatively propagated crops like root or tuber crops have no regular flowering or seed settings and are traditionally maintained in field gene banks. This is expensive, poses risks to diseases and destruction due to other natural calamities and demands space and time.

The preservation of genetic diversity is a vital form of insurance and investment. It requires the prevention from becoming extinction of species and the preservation of as much of variations within species as possible. The fundamentals of plant improvement are genetic variability , recombination, genotype selection and evaluation. Without genetic variability there is no transgressive segregation resulting from recombination and genetic assortment , and therefore, no opportunity for genotype improvement by different selection. Thus , future plant breeding options depend heavily on adequately conserved, broad spectrum germplasm resources to allow gain in future generations and adaptation to changing objectives, environments and management systems.

In vitro collections, which are maintained under short-term storage, require manpower immensely. The *in vitro* plants can be maintained for a long periods or 'long –term storage' by reducing the growth rate which can be achieved by temperature reduction, light intensity reduction, growth regulators use, limitation of minerals supply, addition of osmotic stress agents, or by the combination of any of these methods.

The incorporation of osmotically active compounds such a mannitol, reduction of growth regulators, strength of the nutrients and the use of growth retardants have resulted in the slow growth of cultures (Staritsky and Zandvoort, 1985; Jarret and Gawel, 1991; Malaurie *et al.*, 1993). *In vitro* storage techniques using minimal growth conditions have been applied to apical meristem and shoot cultures of some woody and herbaceous plant species with varying successful storing periods eg., 10 months for *Eucalyptus spp.* (Mascarenhas and Agrawal 1991; Watt *et al,* 2000), 12 months for *Saussarea lappa* (Arora and Bhojwani, 1989), 12 months for apple genotypes (Negri *et al,* 2000) and 60 months for *Populus spp.* (Hausman *et al,* 1994). The short-term conservation of germplasm by the use of mineral oil overlay of cultures has been achieved (Constable and Shyluk, 1994). The mineral oil overlay lowers the oxygen levels. The slow-growth storage of cultures has certain drawbacks, for instance the management of large *in vitro* collections and the possible development of somaclonal variations in cultures.

Materials and Methods

Effect of reduced temperature:

Nodal explants from excised *in vitro* grown shoots of *I. khasiana* and undehisced fruits of *N. tetragona* were inoculated in MS medium and the cultures were incubated at 0°C, 4°C and 8°C (in dark). Control was maintained at 25 ± 2 °C (in the light). Cultures stored at low temperatures were transferred to shoot multiplication media (MS + 2.0 mg/l BAP + 1.0 mg/l KIN for nodal explants; MS + 2.0 mg/l BAP + 0.5 mg/l NAA for undehisced fruits) and kept in culture room temperature 25 ± 2° C and at every one month interval for 8 months, the data was collected on the survival, growth and development. Ten replicates per treatment were taken.

Effect of reduced strength of medium, mannitol and abscissic acid

To study the effect of slow growth treatments on *Ilex khasiana* and *Nymphaea tetragona*, the explants were subjected to the following treatments:

A: ½ strength MS

B: MS+ mannitol (40 gm/l)

C: MS+ mannitol (50 gm/l)

D: MS + mannitol (60 gm/l)

E: MS+ mannitol (70 gm/l)

F: MS + ABA (1.0 mg/l)

G: MS + ABA (2.5 mg/l)

H: MS + ABA (5.0 mg/l)

I: MS + ABA(6.5 mg/l)

J: MS + Sucrose (30 gm/l)

Explants were inoculated on ½ MS and full MS media. Full strength MS medium supplemented with osmotic inhibitor, mannitol, at a range of 40- 70 mg/l and MS with growth retardant ABA at a concentration range of 1.0 - 6.5 mg/l were also used to study their potentialities for slow growth storage of *I. khasiana* and *N. tetragona*. Control was taken on standard shoot multiplication conditions subjected to MS medium containing 2.0 mg/l BAP and 1.0 mg/l KIN for nodal explants and MS+ 2.0 mg/l BAP and 0.5 mg/l NAA for undehisced fruits in the normal culture room. Data were collected every 30 days for 8 months. Ten replicates per treatment were taken. The collected data were subjected to statistical analysis.

The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min and the cultures incubated at 16 h photoperiod of 200 μ mol $m^{-2} s^{-1}$ light intensity.

Results

Effect of reduced temperature

Cultures incubated at different low temperatures showed different responses (Table 5.1). At temperature 0°C, 4°C and 8°C, the explants were found to turn pale green in comparison to the dark green explants at 25 ± 2°C (control).

At 0°C, the culture showed a very low survival percentage after one month. With further storage, none of the cultures survived.

After 1 month, cultures incubated at 4 °C turned pale green with a percentage survival of 20. The percentage survival gradually increased up to 6 months with 60 percentage survival and the explants tuned green in *I. khasiana*. In case of *N. tetragona,* the percentage survival was found to be optimal with 50 after 5 months of storage and thereafter percentage survival in both the cases declined. The explants lost colour with further storage and eventually died.

Thus, of all the temperature treatments, explants could be preserved for a period of 5- 6 months at 4 °C with percentage survival of 60 and 50 in the explants of *I. khasiana* and *N. tetragona* respectively.

At 8° C, the explants of both the plants resulted in low percentage survival and turned pale green in colour after storage. Both the nodal segment of *I.khasiana* and undehisced fruits of *N. tetragona* 30% survival after 8 months of storage. The survival percentage subsequently declined with further storage.

Effect of slow growth treatments

***I. khasiana*:**

Out of different minimal or slow growth methods (1/2 MS, MS + mannitol, MS + ABA), ½ strength nutrient MS was found to be the most suitable for regeneration of explants after storage . The explants remained viable and regenerated after 6 months of storage. The regeneration percentage was reduced to 50 after 7 months which further declined to 10% after 8 months in *I .khasiana* (Table 5.2 A). The explants regenerated shoots with healthy green leaves. The treatment H containing the retardant ABA (5.0 mg/l) in MS medium was also suitable for reducing the growth of explant with percentage survival of 60% after 6 months which further reduced to 40 and declined to 10% by the end of 8 months. However, the regenerated shoots after storage developed with necrotic leaves which became healthy when brought back to normal conditions of growth on MS medium containing 2.0 mg/l BAP and 1.0 mg/l KIN (Photoplate 5.1A). The treatment J :MS+30% sugar at reduced light intensity failed to regenerate after storage of cultures.

The treatment containing mannitol in MS medium was not suitable for slow growth of explants during storage. The explants became pale with low percentage survival and ultimately died in course of storage.

***N. tetragona* :**

In case of *N. tetragona*, the storage of explants, the percentage survival was was optimum in treatment D containing MS+ mannitol (60 gm/l) (Table 5.3). In this treatment 50% survival of the explants was recorded and eventually the explants regenerated shoots with leaves when cultured in MS medium containing 2.0 mg/l BAP 0.5 mg/l NAA (Photoplate 5.2 A).

In treatment A : ½ MS medium, the percentage survival was 50 after 5 months of storage with green leafy shoots (Table 5.3). However, with ABA in the medium the results were not satisfactory. Here the percentage survival was very low (below 30%) and explants turned pale and gradually died (Photoplate 5.2 B).

Incorporation of mannitol in the medium resulted in storage upto 5 months. The maximum survival of the explants was recorded at 60 gm/l of mannitol in MS medium after 5 months of storage. Beyond 5 months, it was found that the survivability of the explants decreased markedly.

Reducing the light intensity in the MS+30% Sugar resulted poor regeneration percentage and ultimately explants died.

Discussion

In vitro conservation aims to maintain germplasm in a relatively stable form under more or less defined nutrient conditions in an artificial environment.

The principle of *in vitro* storage method is to reduce the need of frequent subcultures and preserve the unique genetic constitution of germplasm. The widely applied methods to reduce the growth rate for convenient germplasm storage involve reducing the mineral supply, temperature reduction, light intensity reduction, incorporation of growth retardants, addition of osmotic stress agents or by the combination of any of these methods.

In present study, *in vitro* shoots of *I. khasiana* could be preserved at 4°C in dark for 6 months and the increase of temperature to 8°C resulted in the pale colour and death of the cultures. Similarly, explants in *N. tetragoan* could be preserved up to 5 months with 50 percent survival. However, at 8°C the survival rate was reduced to 40 percent. Upadhyay *et al.* (1989) reported that 70% of the shoots of *Picrorhiza kurroa* could be stored for 10 months at 5°C in dark, remained viable. Similar results of cultures have been reported to be stored at reduced temperatures in the dark (Marino *et al,* 1985; Arora and Bhojwani, 1989); under reduced light conditions (Banerjee and Delanghe, 1985; Upadhyay et al., 1989; Roca, 1990) and under low temperature (Wilkins *et al.,* 1991).

Bapat and Rao (1988) reported that encapsulated embryo of *Santalum album* germinated after storage at 4°C for 45 days with less than 5% survival rate. The relatively short survival period and multiplication capacity of the shoots stored at 8°C was noted in both the plant species. This is, in agreement, with Watt *et al,* (2000) who observed relatively short survival period of shoots stored at 10°C. Pattnaik and Chand (2000) also reported that encapsulated buds of *Morus* species could be stored for 60-90 days at 4°C.

However, Maruyama *et al.* (1997) were successful in retrieving plantlets from 70-90% of the alginated shoot tips of three tree species following a 6-12 month storage at 12-25°C.

In vitro storage of germplasm under minimal or slow growth conditions has been used for many plant species (Chaturvedi *et al.*, 1991; Mascrenhas and Agrawal, 1991; Hausman *et al.*, 1994; Negri *et al,* 2000).

In the present study, ½ strength MS medium was found to be suitable where explants of both *I. khasiana* and *N. tetragona* remained viable at 4°C in dark for a period of 5-6 months. Berjak *et al*(1996} also reported that *in vitro* shoots of *Eucalyptus* species could survive for 6 months when maintained at 4°C with low light intensity of 4μmoles $m^{-2}s^{-1}$ on ¼ th strength MS. However, in contrast, · Bonnier *et al* (1997) observed growth inhibition in reduced nutrient supply. The use of 60 gm/l mannitol in MS was found to be suitable for explant regeneration of *N. tetragona* with 50 percent survival, whereas shoots of *I. khasiana* turned pale and subsequently died. The use of mannitol was also reported as pre-growth media additive for preservation studies (Pritchard *et al.*, 1986; Love *et a.*, 1987). Espinoza *et al*(1986) also reported osmotic stress that leads to reduction in the rate at 25°C. Similar result of successful freeze-hardening of embryos using a high concentration of osmoticum (mannitol) was reported to give an increased survival rate (Delvallee *et al,* 1989). However, the incorporation of ABA (5.0 mg/l) in MS medium was promising for the shoots of *I. khasiana* which survived for 6 months whereas in case of *N. tetragona,* shoots could not survive. Although the shoots of *I. khasiana* survived for 6 months in storage with the incorporation of ABA in the medium, some leaves developed necrotic spots with lesser number of shoots /explant in comparison to control. Wakabayashi *et al.* (1991) suggested that suppression of growth by ABA application could be the result of inhibition of cell elongation as well as cell wall synthesis. ABA concentration, in our study, seems to affect the shoot growth of *I. khasiana* greatly at 5.0 mg/l. Watt *et al.* (2000) also reported that shoot of *Eucalyptus grandis* could be stored for 10 months in the treatment of 10 mg/l ABA in ½ MS . But incorporation of mannitol in the medium did not evoke positive response.

In vitro storage under minimal growth techniques have been applied to meristem and shoot cultures of some forest species with varying successful periods, e.g., 10 months for *Eucalyptus citriodora* (Mascarenhas and Agrawal, 1991) and 60 months for *Populus* spp. (Hausman *et al.*, 1994).

Therefore, the present study shows that the nodal explants of *I. khasiana* and the embryo from undehisced fruits of *N. tetragona* could be stored at the low temperature of 4°C for 6 months and 5 months respectively. The reduction in the nutrients of MS medium to half also promoted the storage period by retarding the growth in both the species studied.

Table 5.1. Percentage survival / or differentiation of shoots after storage at low temperature in dark

Temp. (°C)	**Explants**	**Survival % (in months)**							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
* 25 ± 2°C (Control)									
0	NS	10±0.9	0	0	0	0	0	0	0
	UF	10±0.8	0	0	0	0	0	0	0
4	NS	20±1.6	30±1.8	30±1.0	40±0.5	50±1.4	60±1.7	40±1.2	20±0.9
	UF	10±1.2	20±1.2	30±1.9	40±2.0	50±2.4	20±1.3	10±0.8	0
8	NS	10±1.8	10±1.4	20±1.5	30±1.2	30±0.9	20±0.8	10±0.6	0
	UF	10±1.6	10±1.0	20±0.9	20±1.2	30±0.8	20±0.7	10±0.5	0

± SD

*25 ± 2°C (control) result 90-100% survival from the first month onwards

NS - nodal segment of *I. Khasiana*

UF - undehisced fruits of *N. tetragona*

Table 5.2. Percentage survival and regeneration of nodal explants of *I. khasiana* given slow growth treatments

Treatments	**Survival % (months)**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A. ½ strength MS medium	20±1.5	30±2.1	40±2.4	5±1.8	60±2.1	70±2.5	50±1.4	10±1.0
B. MS + mannitol (40 gm/l)	0	10±0.9	10±1.2	20±1.8	20±1.6	30±0.8	10±1.1	0
C. MS + mannitol (50 gm/l)	0	10±0.8	10±1.2	20±1.3	30±1.4	30±0.9	10±0.8	0
D. MS + mannitol (60 gm/l)	10±1.2	10±1.4	20±1.6	20±1.9	30±2.5	30±1.2	10±0.9	0
E. MS + mannitol (70 gm/l)	0	10±0.8	10±0.7	20±0.5	30±1.2	30±1.5	10±1.4	0
F. MS + ABA (1.0 mg/l)	0	10±1.2	20±0.8	30±0.5	40±0.9	20±0.5	10±0.8	0
G. MS + ABA (2.5 mg/l)	10±1.3	20±1.5	30±1.2	30±1.3	40±1.3	40±1.4	20±1.7	0
H. MS + ABA (5.0 mg/l)	20±1.4	30±0.8	40±0.7	40±1.4	50±2.1	60±1.8	40±1.9	10±0.5
I. MS + ABA (6.5 mg/l)	10±1.3	20±1.4	30±0.9	40±0.4	40±0.5	50±0.8	40±1.2	10±1.3
J. MS + sugar (30%)	0	10±1.2	20±1.4	30±1.6	20±1.5	10±1.4	10±1.6	0

± SD

A to F - treatments were in a 16 h photoperiod at 25 ±2°C in 200μmol $m^{-2}sec^{-1}$

J - treatment was at constant 50μmol $m^{-2}sec^{-1}$ and 8°C

Explants used : nodal explants

Data collected upto 8 months

Table 5.3. Percentage survival and regeneration of embryo from undehisced fruits of *N. tetragona* given slow growth treatments

Treatments	Survival (months)							
	1	2	3	4	5	6	7	8
A. ½ strength MS Medium	10±1.8	20±0.9	30±1.5	40±1.4	50±1.8	30±1.6	30±1.5	10±1.2
B. MS + mannitol (40 gm/l)	10±1.4	20±1.3	20±1.7	30±1.8	40±1.7	20±1.9	10±2.1	0
C. MS + mannitol (50 gm/l)	10±1.8	20±1.9	30±0.9	30±0.8	40±1.8	30±2.1	10±1.5	0
D. MS + mannitol (60 gm/l)	10±1.9	20±1.7	30±1.8	40±1.7	50±1.5	30±2.4	10±0.9	0
E. MS + mannitol (70 gm/l)	10±1.6	10±1.4	20±1.3	30±1.9	40±1.7	20±1.8	10±2.4	0
F. MS + ABA (1.0 mg/l)	0	10±1.2	20±2.1	20±1.7	20±1.6	10±0.9	10±0.8	0
G. MS + ABA (2.5 mg/l)	0	10±0.9	20±0.8	30±1.5	30±1.6	20±1.4	10±1.2	0
H. MS + ABA (5.0 mg/l)	0	10±2.1	20±1.9	20±1.8	30±1.7	20±0.9	10±1.8	0
I. MS + ABA (6.5 mg/l)	0	10±1.7	20±2.1	30±1.8	30±1.5	10±1.6	10±2.1	0
J. MS + sugar (30%)	10±1.5	10±1.3	20±1.6	30±1.4	20±1.8	20±1.4	10±1.9	0

± SD

A- F treatments were in a 16 h photoperiod at 25 ±2°C in 200μmol $m^{-2}sec^{-1}$

J treatment was at constant 50μmol $m^{-2}sec^{-1}$ and 8°C

Data collected upto 8 months

Photoplate 5.1

A - B: Multiple shoots after retrieval from $4^{o}C$ cold storage on ½ MS medium + BAP (2.0 mg/l) + KIN (1.0 mg/l)

A: Multiple shoots with necrotic leaves on MS +5 mg/l ABA

B: Multiple shoots with necrotic spots on ½ MS


A
B

Photoplate 5.2

A - B: Plantlet regeneration from embryos in *N. tetragona* after retrieval from 4 °C cold storage in MS medium + BAP (2.0 mg/l) + NAA (0.5 mg/l) and MS+ABA (2.5 mg/l) respectively.


A
B

CHAPTER VI: HARDENING, TRANSFER AND ESTABLISHMENT OF *IN VITRO* RAISED PLANTS OF *ILEX KHASIANA* AND *NYMPHAEA TETRAGONA*

Introduction

The overall success of tissue culture raised plants depends on successful hardening and transplantation in the field. After achieving the *in vitro* raised plants, they a re to be carefully hardened and acclimatized. The readily available nutrient supply and controlled environmental conditions do not necessitate anatomical and morphological conditions in micropropagated plants during *in vitro* cultures. Plantlets which a re cultured *in vitro* on agar-based media, in a water-deficit atmosphere, wilt rapidly on transfer to normal green house or field conditions. Poor water uptake and rapid loss of water through transpiration (Grout and Aston, 1977) my lead to high mortality rate unless plantlets are acclimatized by gradual stages to reduced humidity and increased light intensity (George and Sherrington, 1984). The problem of water relations are compounded by damage to the delicate shoots and roots during transplantation (Debergh and Maene, 1981). The micropropagated plants lose excessive water, resulting in reportedly lower survival rates in the field. The scarcity or poor availability of ground water also hampers the successful transplantation of *in vitro* raised plants. So, the rate of survival of micropropagated plants in field conditions goes beyond the expectation. Bhojwani and Razdan (1983) have stressed on high humidity conditions during the initial days for successful transplantation.

Temperature plays an important role in the survival rate and growth of transplanted plants. This shock is much more severe in the cold region where temperature during winter drops to sub-zero levels. Moreover during summer, plants are exposed to high irradiance and temperatures (30-40^{o}C), and low humidity. Thus the transfer of *in vitro* raised plantlets to pots or field/exposed environmental conditions need a careful step-wise procedure for better survival.

The type of composts plays an important role in the survival rate of transplants of *in vitro* raised plantlets. Moreover, good drainage and sufficient aeration of roots are essential factors for the luxuriant growth of the transferred plants.

In order to facilitate hardening and to raise the percent survival of *in vitro* raised plants in the field, Palni *et al.*, (1994) have developed a simple method aptly called 'polypit' or poor man's growth chamber to facilitate hardening and make it cost-effective. It was found to be almost like a high tech microcomputer controlled growth chamber (Palni *et al.*, 1994; Vyas *et al.*, 1999). A 'polypit' of approximately 2 x 1.2 x 1.0 m dimension, covered on top with polythene sheet of 162 μm thickness maintained high relative humidity, elevated CO_2 level and optimal high temperature during winters and low temperature during summers.

By using 'polypit' several works on hardening and acclimation of *in vitro* raised plants were successfully achieved with high rate of survival. Micropropagated plants of several species of tea and oak were transferred from the culture room to small pots containing soil and maintained under high humid conditions by covering with polythene bags and kept in the 'polypit'. About ten days later, the polythene bags were removed resulting in decrease in humidity and the plants gradually acclimatized with 3-4 weeks in the same 'polypit' house. After 6 months, a high survival rate of inoculated plants was achieved. (Palni *et al.*, 1994; Bisht *et al.*, 1998; Vyas *et al.*, 1999).

Right stage of transplants , suitable compost, moisture and other physical factors greatly affect the survival rate of plants. In the present chapter, successful hardening of the *in vitro* raised regenerants of *I. khasiana* and *N. tetragona*, their transfer and establishment under glasshouse conditions have been studied.

Materials and Methods

Healthy *in vitro* obtained plantlets were used for transplantation studies. Plastic and or earthen pots were used for the present study. Minute holes were pierced at the bottom of the pots using a hot needle. These were then filled 3/4th with different compost combinations and moistened for planting convenience. Potting mixtures used for the transplantation were :

i) forest black soil and sand (2:1)

ii) forest black soil, vermiculite and sand (2:1:1)

iii) Sand and black soil (2:1)

iv) Vermiculite alone

Well developed rooted shoots measuring 3-5cm in height were carefully removed from the culture vessels by spatula. Agar adhering to the roots was removed carefully without damaging the roots. Thereafter plantlets were dipped in 0.1 % Bivastin or Diethane solution for 2 min to minimize the microbial infection. Plants were then carefully placed into the compost and covered with plastic bags. Transplanted plantlets were fed with 1/4th MS basal salt liquid medium every alternate day for the initial first week and once a week in the 2nd week for one month. The average minimum and maximum temperature of the glasshouse at the time of transplantation were 16^{o}C and 23^{o}C. The relative humidity was maintained at 70-80%.

In case of plantlets of *N. tetragona*, the compost mixture was allowed to settle down in the plastic tub containing water. The other conditions of hardening and transfer remained the same for both the plant species.

Results

Of the various composts used, the combination of black soil of forest, vermiculite and sand (2:1:1) was found to be the best substratum for the survival and healthy growth of the *I. khasiana* plantlets wherein 60% survival rate was recorded (Table 6.1). The compost having black soil and sand (2:1) reduced the survival rate of the transferred plantlet to 50%. In case of *N. tetragona* plantlets, sand and black soil (2:1) was the most suitable compost for the healthy growth and development (Table 6.2). The compost comprising vermiculite alone did not support good growth and survival of plantlets of both the plant species. Supplying the plantlets with 1/4th MS basal salt liquid medium proved to be beneficial for their healthy growth. Keeping the plantlets in shade initially improved their survival rate and growth. In three to four weeks time the plantlets were hardened in respective suitable substrata (Photoplate 6.1 and 6.2). 60% of the hardened plants derived from nodal explants of *I. khasiana* when transferred to field survived. Callus derived plants showed less survival rate (Table 6.3). In case of *N. tetragona,* embryo- derived plants (Photoplate 6.3 A,B) showed better survival in plastic tub as compared to plants obtained from rhizome culture (Photoplate 6.2 A, B).

Discussion

The successful transplantation depends on the suitable size and growth of the regenerants. It is well accepted that healthy regenerants are easier to transplant and are less susceptible to diseases and mechanical injury. The transferred plantlets had a healthy and vigorously growing root system which assured higher establishment and growth. The deflasked regenerants are often plunged directly into a fungicide solution before being planted in pots and the practice is believed to prevent damping off (Sessler, 1978). At times, however, fungicide solutions are harmful and the treated plantlets remain permanently stunted (Kang, 1979).

Compost containing black soil, vermiculite and sand in the ratio of 2 : 1 : 1 was found to be the best for healthy growth and development of transplanted plants of *I. khasiana.* However, the greater ratio of sand to black soil (3 : 1) was the best for the healthy growth and survival rate of *N. tetragona* as it might have facilitated the proper drainage and aeration for root respiration. Xie and Hong (2001) successfully transplanted the *in vitro* raised plantlets of *Acacia mangium* in the pots containing peat and white sand in the ratio 3 : 1. *In vitro* plantlets of *Pinus strobus* were successfully transplanted using compost containing perlite : peat moss: vermiculite (1 : 1 : 1) by Tang and Newton (2005). Maintenance of high humidity during initial transplantation period was quite promising to prevent desiccation of plants. This was achieved by covering the plants with plastic bags. Moreover, reducing the light period at initial stages was necessary for the healthy growth of the transplanted plants. Plantlets cultured *in vitro* are highly susceptible to desiccation once transferred to soil. Partial defoliation of the plantlets at the time of transplantation is reported to be beneficial in certain cases (Bhojwani, 1980; Tisserat, 1981). However, in the present study, there was no need to remove the leaves as they were few in number. However, the desiccation problem was not there in case of *N. tetragona* as the plants were transferred in water. But the problem of contamination was found on transfer in case of plantlets derived through rhizome bud cultures. Direct exposure to sunlight was harmful to the transferred plantlets which could be due to the increase in temperature at the leaf surface. The rate of plantlets survived was more if the plants were subjected to shade for 3-4 days at initial transplantation stage. Supplying 1/4th strength MS salt solution was found to be beneficial as it supplied essential nutrients to the developing plantlets. Similar reports of supplying 1/4th MS salt solution to the potted plants during hardening of *in vitro* plantlets of wild strawberry has been reported by Bhatt and Dhar (2000). Survival of transferred plants depends on their ability to carry out photosynthesis and withstand water loss. *In vitro* plantlets have the characteristics of less or no photosynthetic pigments, malfunctioning of stomata and marked decrease in epicuticular waxes (Bhojwani and Dhawan, 1989) that lead to the desiccation and death of transplanted plant. Many studies have shown that inclusion of triazoles e.g. paclobutrazol in the rooting media is promising in the protection against different stresses such as chilling, heat shock, water-logging and drought stress (Kraus and Fletcher, 1994; Gilley and Fletcher, 1997 ; Panaia *et al.*, 2000). The supply of diluted nutrient solution to the hardened orchid plants for one monhth has been reported to be beneficial (Kumaria,1991; Kumaria and Tandon,1994)). The plants were hardened within four weeks of transferring them in the pot. The hardened embryo derived plants *N. tetragona* started flowering on being transferred to the plastic tub after 2½ -3 months.

Table 6.1. Hardening of *in vitro* grown plantlets of *I. khasiana* in different potting substrata

Substratum used	% survival	Growth
i) Black soil + sand (2:1)	50	++
ii) Black soil + vermiculite + sand (2:1:1)	60	+++
iii) Sand + black soil (3:1)	40	-
vi) Vermiculite alone	30	+

- poor growth; + fair growth; ++ good growth; +++ best growth
Data collected after 4 weeks of transfer.

Table 6.2. Hardening of *in vitro* grown plantlets of *N. tetragona* in different potting substrata

Substratum used	% survival	Growth
i) Black soil + sand (2:1)	30	+
ii) Black soil + vermiculite + sand (2:1:1)	40	++
iii) Sand + black soil (3:1)	60	+++
iv) Vermiculite alone	30	+

+ fair growth; ++ good growth; +++ best growth
Data collected after 4 weeks of transfer.

Table 6.3. The percentage survival rates in the field/plastic tub of *in vitro* raised plantlets of *I. khasiana* and *N. tetragona* after subsequent hardening and acclimatization (Data recorded : after 4 weeks)

Source		**Days of observation**	**No. of plants transferred**	**Survived**	**Infected**	**% survival**
I. khasiana	Plantlets obtained by direct multiple shoot formation on nodal explants	7	10	2	8	20
		14	10	4	6	40
		21	10	6	4	60
		28	10	6	4	60
	Plantlets obtained through callus on leaf explants	7	10	1	9	10
		14	10	2	8	20
		21	10	4	6	40
		28	10	4	6	40
N. tetragona	Plantlets obtained from rhizome explants	7	10	2	8	20
		14	10	4	6	40
		21	10	5	5	50
		28	10	5	5	50
	Plantlets obtained through embryo culture	7	10	2	8	20
		14	10	5	5	50
		21	10	7	3	60
		28	10	7	3	60

Photoplate: 6. 1

A & B: Hardening of *in vitro* raised plants of *I. khasiana*

A: Potted in green house

B: 3 months old plants in pots


A
B

Photoplate: 6.2

A - B: Rhizome-derived hardened plants of *N. tetragona*

A: Plants after 2 weeks

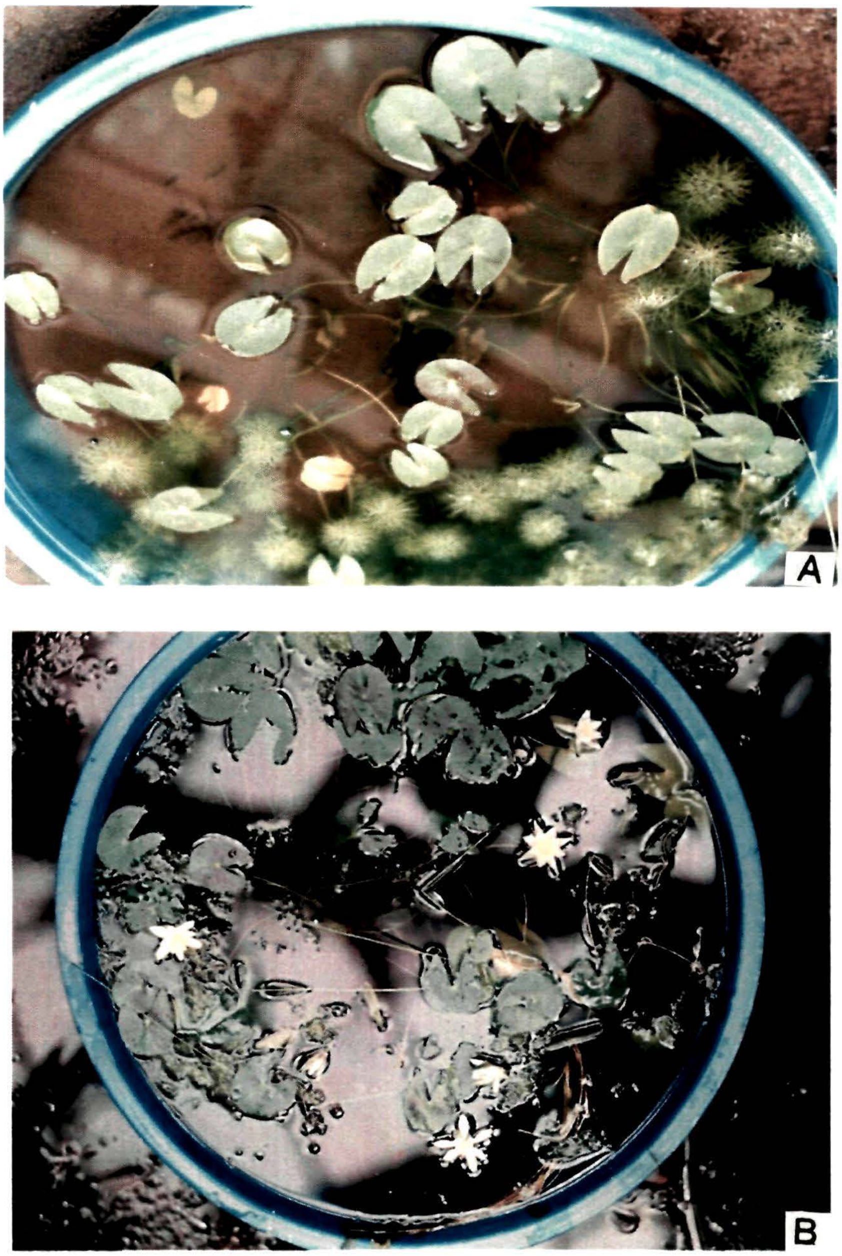
B: Hardened unhealthy plants (4 weeks old)

Photoplate 6.3

A - B: Embryo derived plantlets of *N. tetragona* in the tub

A: Hardened healthy plants 6 weeks old

B: Flowering plants after 2 ½ months


A
B

CHAPTER VII: SUMMARY

India with a large reservoir of diverse plant species is designated as one of the 12 mega diversity hot spots in the world. Home to numerous medicinal and variety of plants, India has both Alpine and Tropical plant species because of its varying climate. From time immemorial man has relied on plants for food and treatment of various kinds of ailments. Population explosion has resulted in the need for land for agriculture, urbanization and other activities. Deforestation of tropical as well as temperate forests on a large scale, shifting cultivation and soil erosion are some of the main factors that have contributed to the depletion of natural biodiversity. There is an increasing pressure on the plants for human survival and economic well being throughout the world. The overexploitation of plants for medicinal and commercial purposes has also degraded the natural genetic resources. Besides, rapid industrialization coupled with increased human activities have resulted much to the shrinking of the natural plant resources of biodiversity.

Due to such rapid destruction of forest resources by mankind, numerous plant species have already become extinct; many more are becoming rare/endangered in their natural habitats. In fact, along with such plants, *Ilex khasiana*, a holly tree and *Nymphaea tetragona* an aquatic lily herb have also been placed in the list of rare and endangered plants. *Ilex khasiana* is used during Christmas decorations. It is one of the few forest trees of Khasi Hills, Meghalaya and has enormous potential for maintaining landscape and healthy environment (or ecosystems) of hilly regions. The main problem with this tree is its ill-rudimentary developed embryo and seeds bearing such embryos can hardly germinate in nature. On the other hand, it cannot be vegetatively propagated through cuttings. In the past, *I. khasiana* was abundantly present throughout Khasi Hills but at present it is restricted to a few pocket areas of this region. *Nymphaea tetragona,* an aquatic lily has a very low population confined to a single place at Nongkrem Smit pond, Shillong and is on the verge of extinction. This water lily is valuable ornamentally for its showy flowers. The rhizome of the plant is locally used to cure acute diarrhoea and to join bone-fracture.

Keeping in mind the ever increasing threat to plant diversity of the Northeastern region, there is an urgent need to conserve these two important rare and endangered plant species. The proposed study was undertaken with the following objectives:

1. Micropropagation of *I. khasiana* and *N. tetragona* using various explant sources, media, and culture conditions.
2. Developing complete protocol for mass multiplication of these plants.
3. Developing short to medium-term *in vitro* storage methods.

The main methods adopted for the successful completion of the present work are summarized below:

- Explants like apical buds, nodal stem segments, leaf discs, rhizome buds were tested in different seasons for their best performance on the culture media.

- For checking browning of explants from mature trees, various pre-treatments like ascorbic acid 50 mg/l, PVP (0.5%), citric acid (75mg/l), charcoal (1-2%), chilling, distilled water and serial transfer of explants at 2, 4, 8, 12, 24, 48, 96 h intervals were done for maximum survival of the explants on the culture medium.
- Detergents like teepol and soap water were used with different concentrations and varying period of time for thorough cleaning of the explants.
- For the surface-sterilization of explants, the sterilants like $HgCl_2$ solutions, ethanol 70% and Savlon were used with different concentrations and time period.
- Different nutrient media like MS, LS, Nitsch and White were tested for optimising best growth and development of explants in the culture.
- Effects of different cytokinins like BAP, KIN and Zeatin were recorded for the direct multiple shoot bud induction.
- Effect of cytokinin-auxin ratio on the induction of multiple shoots was seen.
- For the initiation of callus from leaf of *I. Khasiana*, effects of different auxins alone and combinations of auxin-cytokinin were observed.
- Induction of caulogenesis from callus was tried in the medium treated with various concentrations of growth hormones.
- For cold storage, *in vitro* obtained shoots of *I. khasiana* and of *N. tetragona* were kept at 4 ^{0}C in dark for a period of upto 8 months and the regeneration capacity and duration of survival were tested at monthly intervals.

- Various pre-treatments like reduced supply of nutrients, low temperature treatment, addition of ABA, addition of mannitol or sugar for low osmoticum were tested for the optimal cold storage conditions.
- Easy and cost effective methods for the successful hardening and acclimatization of *in vitro* obtained plantlets were investigated.

Main Outcomes of the Present Study:

The important outcomes of the present research work are summarized below:

- The suitable season for explant collection for direct multiplication of shoot regeneration was found to be April to July in both the cases.
- For surface sterilization, initial treatment of the explants with 0.5% teepol detergent for 30 minutes followed by Na OCl (10%) or $HgCl_2$ (0.01-0.1%) solution for 10-15 minutes, 70% ethanol treatment for 10- 30 seconds and inoculation to the medium showed maximum survival of the explants in the culture medium.
- In case of *I. khasiana*, the browning of the explants collected from mature trees, could be checked, by transferring explants serially on to the fresh medium with 0.5% PVP at 2,4,8,12,24,48,96 h intervals.
- To reduce/overcome the problem of browning and death of the explants from mature trees of *I. Khasiana,* the *in vitro* raised seedlings from seeds were used. The percentage of seed germination was recorded to be only 5-10 % after 2 ½ months.
- The most suitable explants for direct shoot multiplication of *I. khasiana* was the nodal explant from the young seedlings. The average number of shoots

obtained was 10-11 shoots per explant in MS medium containing cytokinins BAP (2.0 mg/l) + KIN (1.0 mg/l).

- In case of *N. tetragona*, cultured rhizome buds proliferated multiple shoots in MS medium incorporated with BAP (2.5 mg/l) + IAA (0.5 mg/l). Within 4-6 weeks an average number of 3-4 shoots were produced.
- The premature embryo culture of *N. tetragona* was successfully carried out and the number of multiple shoots was 12 per embryo in MS medium incorporated with BAP (2.0 mg/l) + NAA (0.5 mg/l).
- For *in vitro* rooting, MS half- strength medium supplemented with IBA (2.0 mg/l) was found to be optimal in case of *I. khasiana* and hormone-free MS medium in *N. tetragona.*
- Among different explants used, leaf discs of *I. khasiana* responded very well and within 4 weeks callus induction could be obtained.
- MS medium supplemented with 2, 4- D (2.0 mg/l) + KIN (0.5 mg/l) was the optimum for the induction of callus.
- Shoots could be induced from the callus of *I. khasiana* in ½ MS medium containing a combination of 1.5 mg/l BAP and 0.5 mg/l IBA.
- For elongation and development of plantlets from callus, gradual removal of 2,4-D from the medium was necessary.
- The cold storage period of *in vitro* shoots of *I. khasiana* was upto 6 months whereas in case embryo of *N. tetragona* the storage period was 5 months.
- Among the different effects of minimal growth conditions on the survival and multiplication of shoots after, the treatments of reduced ½ strength MS and

5.0 mg/l ABA in MS medium showed the best survival and multiplication rate.

- Plantlets produced by direct multiple shoot induction showed 60 % survival after hardening in *I. Khasiana.*
- Plantlets produced indirectly through callus showed 40 % survival after hardening.
- Plantlets obtained directly from rhizome and embryo explants of *N. tetragona* showed 50 % and 60 % survival respectively after hardening.

Conclusion and limitation

Plant tissue culture technique, an integral part of biotechnology deals with the mass propagation and conservation of elite clones and varieties. Recent advances on plant tissue culture coupled with genetic engineering made possible to obtain GMO's (Genetically Modified organisms) to increase the overall productivity, with quality and disease/drought resistance of desired traits. Callus mediated plantlets have potential for somaclonal variations for commercially improved plants. The recent trend of emerging plant tissue culture along with molecular biology can provide integrated and valuable information particularly in understanding the genetic control of each development of callus, caulogenesis and plantlet development. The protocols developed for mass multiplication could easily be adopted for the large scale propagation of important plants with a view to sustainable conservation.

CHAPTER VIII: REFERENCES

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BIODATA

Name	:	SHRI JITEN CHANDRA DANG
Fathers Name	:	Late KIRSING DANG
Date of Birth	:	01. 03. 1963
Present Address	:	Senior Lecturer, Botany Department Don Bosco College, Tura Meghalaya Pin 794 002
Permanent Address	:	Goipani Village P. O. Koomsang, Doomdooma District Tinsukia, Assam
Sex	:	Male
Nationality	:	Indian
Category	:	ST
Educational Qualifications	:	

Name of the examination	**Board/University**	**Division**	**Year of passing**
HSLC	Board of Secondary Education, Assam	II	1978
Pre-University	Dibrugarh University, Assam	III	1980
B.Sc. (Botany Hons.)	North-Eastern Hill University, Shillong	II	1983
M.Sc. (Botany)	North-Eastern Hill University, Shillong	II	1985

J. C. Dang