MICROPROPAGATION AND CONSERVATION OF CRITICALLY ENDANGERED *MANTISIA SPATHULATA* SCHULT. AND *MANTISIA WENGERI* FISCHER (ZINGIBERACEAE) OF NORTH-EAST INDIA

By

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DECLARATION

I do hereby declare that the thesis entitled "**Micropropagation and conservation of critically endangered *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer (Zingiberaceae) of North-East 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 and Dr. Suman Kumaria. The work done is original and no part of the thesis has been submitted for any degree or diploma of any university.

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CERTIFICATE

We certify that the thesis entitled "**Micropropagation and conservation of critically endangered *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer (Zingiberaceae) of North-East India**" submitted by Mr. Sudipta Shekhar Das Bhowmik for the degree of Doctor of Philosophy in Botany of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by him under our 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.

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CONTENTS

CHAPTER I: GENERAL INTRODUCTION

Plant biodiversity is the greatest resource that has been treasured from nature in the form of food, fodder, shelter, biomass etc. required for the survival of humankind. Plant diversity helps to maintain the biophysical systems and chemical balance of the earth and climate thereby stabilizing life system on the planet earth. Out of the 1.7 million plant species that have been described worldwide so far (Groombridge and Jenkins, 2000), more than 8,000 plant species are threatened with extinction, according to the World Conservation Union (Farnsworth, 2007). Indian flora accounts for 10.78% of the global flora. About 80% of the floristic wealth of India is concentrated in North-east (Tandon and Kumaria, 2005). North-east India and the whole Eastern Himalayas is one of the Hotspots of the Globe (Myers *et al.*, 2000). North-eastern India known as the 'cradle of flowering plants' is rich in many unique plant species which are geographically isolated and therefore, endemic to the region. Inspite, of the rich vegetation, flora of this region remains largely unexplored which hinders the full utilization of the plant resources. There are many more species belonging to both flowering and non-flowering plants that are yet to be discovered in the whole North-eastern region (Frodin, 2001; Tandon and Kumaria, 2005). However, the plant genetic resources are getting depleted at a faster rate and several species including many unique and irreplaceable varieties are becoming extinct in recent time. The disappearance of a single species would mean a removal of evolutionary links

that contribute to an understanding of plant life and also the loss of some of their genes that might prove useful in the future. The shrinkage of forest cover due to uncontrolled expansion of human populations for settlements, rapid changing ecosystems, natural calamities like forest fires, landslides etc. and ruthless collection of medicinally and valuable plants for timber and ornamental purposes have led to the crisis in biodiversity management and conservation.

The existing rare and endemic germplasm that is rapidly getting depleted from the wild habitat of North-eastern India needs to be conserved both under *in situ* and *ex situ* conditions. *In situ* conservation involves protection of the environment itself. It is ideal and dynamic approach that allows plants to interact and co-evolve with other components of the ecosystem including insects, animals and microbes. However, *in situ* conservation is costly to maintain and is highly susceptible to natural calamities like forest fires, extreme weather conditions such as rainfall, landslides etc. and damage by diseases and animals. Therefore, in addition to *in situ* conservation, reintroduction of available endemic wild species of this region in various Botanical Gardens is the need of the time for restoration of the rare germplasm.

The total forest area of North-east India is 54% and harbors a large number of important rare and endangered plants. Some of the endemic and rare plants from this region are *Nymphaea tetragona, Magnolia gustavii, Salmonia aphylla, Sarania griffithi, Ilex khasiana, Ilex embeliodes,* several orchids like *Galeola falconeri, Rhynchostylius retusa, Dendrobium hookerianum, Dendrobium bensioae,* etc., insectivorous plants for e.g. *Nepenthes khasiana, Drosera burmanni, Drosera peltata* etc. and many zingibers including *Hedichium calcaratum, Hedichium marginatum,*

Hedichium dekinanum and three species of *Mantisia* (Pence, 1999; Tandon and Kumaria, 2005).

A large number of plants belonging to Zingiberaceae family are concentrated in the North-eastern region of India. The largest family Zingiberaceae comprises 53 genera and over 1200 species. Out of these, around 22 genera with about 200 species are found in India. Among the 22 genera of family Zingiberaceae, *Mantisia*, is the smallest genus with only four species viz. *M. wengeri*, *M. spathulata, M. radicalis* and *M. wardii*. Excluding *M. wardii* which is endemic to Myanmar, the rest of the three species are found only in restricted pockets of North-eastern India. Locally known as 'Aiting', these plants have been used in the past for curing gastro-intestinal ailments and bone injury. In addition to their medicinal properties, species of *Mantisia* possess splendid flowers which are commonly known as 'dancing girl' and can be utilized for commercial purposes in future. The genus, *Mantisia,* was found by Roxburgh in 1808 based on the plants sent to Britain from Calcutta Botanical Garden. The plants were collected by Roxburgh during the year 1801 from the Chittagong forest now southern part of Mizoram, India (Dam *et al.*, 1997).

Mantisia, along with other genera such as *Globba*, *Kaempferia, Curcuma, Ammomum* etc. requires a more detailed study as the vegetative and flowering phases occur alternately at two different time intervals. The vegetative and floral characteristics between the species of genus *Globba* and *Mantisia* are almost similar. Hence, the possibility of identifying the species of *Mantisia* among other *Globba* species in wild habitats during the blooming period depends largely on the floral characteristics. A characteristic difference between the two genera is the of position inflorescence. Unlike all the species of *Globba* which bear flowers at the terminal end

of the vegetative shoot itself, separate floral spikes bearing numerous splendid flowers of *M. spathulata* (pale violet with yellow lip) and *M. wengeri* (yellow colour) appear during early monsoon before the onset of vegetative shoots. During the monsoon season the connectivity in the remote areas often becomes difficult due to excessive rainfall and landslides. Hence, these species remained unrecorded in their natural habitat for the past two decades. The plants have become endangered in their natural habitat and are listed in the Red Data Sheet of Indian plants (www.envfor.nic.in/bsi/research.html). The rarity of *M. wengeri* has reached a critical level and has been included in the national priority list for its recovery (Department of Biotechnology, New Delhi) (Ganeshaiah, 2005). Due to its endemism and rarity, the Government of Mizoram, India has declared the genus *Mantisia* as the 'State Flower'.

The collection and conservation of these two species viz. *M. spathulata* and *M. wengeri* is of prime interest for the recovery and restoration of the limited germplasm available in nature. *In vitro* multiplication provides a rapid alternative method for the propagation of such endangered plants irrespective of seasonal, biotic and abiotic limitations. Plants which are totipotent in nature can be regenerated from isolated cells if they were provided with optimum nutrition and required conditions. This concept of totipotency was first introduced for raising the axenic cultures of *Lamium purpureum* by Haberlandt in 1902. The concept was strengenthened when Gautheret (1940) published the first report on organogenesis in two species of tree tissue cultures. He induced adventitious buds in cambial cultures of *Ulmus campestris* on a medium supplemented with sugars. On the basis of this concept many works have been carried out for the rapid multiplication of many important rare and endangered zingibers such as, *Zingiber officinale* (Hosoki and Sagawa, 1977),

Curcuma longa (Nadgauda *et al.*, 1978), *Alpinia galanga* (Borthakur *et al.*, 1999), *Zingiber petiolatum* (Prathanturarug *et al.*, 2004), *Amomum krervanh* (Tefera and Wannakrairo, 2004), *Mantisia spathulata* and *Mantisia spathulata* (present study: Tandon *et al.*, 2007a; Bhowmik *et al.*, 2009). Clonal propagation through tissue culture is popularly called micropropagation. In majority of species, plantlets are produced from young juvenile material. Rejuvenation of a mature plant is difficult because of 'aging effect' though it has been achieved in several systems (Bajaj, 1986a). The growth controls that operate in an intact plant can be broken down or eliminated under *in vitro* conditions, leading to profuse production of shoots from a single initial explant. The shoots can be separated and rooted to give rise to entire plantlet. Sometimes rooting occurs simultaneously along with the emergence of shoots. Generally micropropagation is approached in three ways i.e somatic embryogenesis, adventious bud differentiation and enhanced axillary bud break. Somatic embryogenesis is the development of embryos from somatic tissues of the plants. The somatic embryos can be produced directly on the explants or indirectly from callus or cell suspension cultures. Somatic embryogenesis from immature and mature zygotic embryos was reported for the first time in a conifer, *Picea abies* (Chalupa, 1985; Hakman *et al.*, 1985). Clonal propagation via adventious meristems involves the induction of unipolar shoots on explants followed by shoot excision and induction of root meristems. The process of adventive meristem production can follow two major developmental sequences- direct or indirect. Initiation of shoot meristems directly from the primary explant in the absence of an intervening callus phase has been defined as direct organogenesis. Indirect organogenesis involves an initial production of callus tissue from the primary explant followed by the

appearance of meristemoids. Regeneration via indirect organogenesis is limited (Konar and Singh, 1980; Bhatnagar *et al.*, 1983; Kaul and Kochhar, 1987; Gladfelter and Phillips, 1987). However this mode of regeneration is not preferred as variants are regenerated in addition to the parental genomic compliments. Micropropagation using axillary mersistems differs from adventious shoot production in the ontogeny of shoot formation. Regeneration from fascicular meristems has been achieved from juvenile as well as mature tissues.

Plantlets developed *in vitro* wilt rapidly on transfer to normal green house or field conditions. Poor uptake of water and excessive water loss (Grout and Aston, 1977) may lead to high mortality rate unless plantlets are acclimatized by gradual stages to reduced humidity and increased light intensity (George and Sherrington, 1984). The problems of poor water relations are coupled by damage to shoots and roots during transplantations (Debergh and Maene, 1981). The establishment and healthy growth of *in vitro* raised plants in the glass house require suitable conditions of acclimatization and hardening. Different potting mixtures, containers and compost influence the growth of plants (Bose and Bhattacharjee, 1980; Stewart, 1988).

The regenerants arising out of *in vitro* cultures are susceptible to genetic changes due to culture stress and mode of regeneration (Cecchini *et al.*, 1992; Rani and Raina, 1998; Kaushal and Kanwar, 2003). To minimize the risk of any cryptic variations arising out of *in vitro* cultures, it is crucial to assess the genetic uniformity of the *in vitro* plantlets with mother stock before their transfer to the field conditions. The DNA markers offer a more attractive means for examining genetic similarity/dissimilarity since these markers are not developmentally regulated as in case of protein markers. In the recent past, a range of molecular techniques have been

standardized for testing the genetic makeup of tissue-culture-raised plants (Gupta and Varshney, 1999). These include DNA hybridization, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and random amplified length polymorphism (RAPD). The genetic fidelity of offsprings can be established using these techniques. RFLP markers are useful for sampling various regions of the genome and are potentially unlimited in number. However, this is time consuming, costly and requires large amount of tissue samples. On the other hand, RAPD is preferred since small variations in the DNA sequences of two individuals of the same species can be detected precisely (Williams *et al.*, 1993). Usefulness of RAPD in detection of variation has been amply demonstrated in large array of *in vitro* plants by many workers (Rani and Raina, 2000; Preeya *et al.*, 2003; Kaushal and Kanwar, 2003; Mathur *et al.*, 2008).

The plants propagated through *in vitro* cultures have been used for reintroduction to recover the populations of endangered plants in their wild habitats. This has been attempted with several species including *Mammillaria san-angelensis* (Martinez-vazquez and Rubluo, 1989), *Nepenthes khasiana* (Tandon *et al.*, 1990), *Artemisia granatensis* (Clemente *et al.*, 1991), *Agave victoria-reginae* (Rodriguez-garay *et al.*, 1996), *Rhododendron ponticum* (Almeida *et al.*, 2005) and several other orchids viz. *Cyperipedium calceolus*, *Ipsea malabarica* (Ramsay and Stewart, 1998; Gangaprasad, 1999). Sometimes the natural habitat is under the threat of natural calamities such as earthquakes, landslides and heavy rainfall. In such cases, the plants are usually removed from their natural habitats and protected at specialized institutions such as Botanical Gardens, nurseries and gene banks. Botanical Gardens have played an important role in *ex situ* conservation programmes particularly in

acclimatization, rehabilitation, multiplication and judicious exploitation of plant resources. Thus, for conservation, strategies must be developed for the protection of species along with their ecosystems, involving a mix of both *in situ* and *ex situ* methods.

Tissue culture techniques have been extensively used for the rapid multiplication of many important rare zingibers resulting in true-to-type clonal material of their germplasm. However, the goal of conservation also requires preserving the genetic diversity of rare and endangered plants (Pence, 1999). Genetic variations under *in situ* conditions in most of the zingibers are sluggish due to the clonal propagation through rhizomes and lack of seed propagation (Sajina *et al.*, 1997; Miceli *et al.*, 2008). When seeds of endangered species are available, they are generally preferred for propagation, in order to maintain the maximum genetic diversity (Pence, 1999; Tandon and Kumaria, 2005). Most of the endangered species produce seeds, but in some cases they are few in number such as *Delphinium malabaricum, Adhatoda bwddomei,* etc. (Agrawal *et al.*, 1991) or they may be difficult to germinate as in *Trillium persistens* (Pence and Soukup, 1995). When very few seeds are available, seeds are germinated under *in vitro* conditions that are used to produce aseptic seedlings so as to produce shoot tips and nodes for micropropagation. This approach has been tried in a number of species such as *Limonium* spp. (Martin and Perez, 1995), *Nepenthes khasiana* (Latha and Seeni, 1994; Nongrum *et al.*, 2009) and *Gentiana lutea* (Momcilovic *et al.*, 1997). When conventional procedures, such as stratification, fail to break seed dormancy or the rate of seed germination is very low, embryo culture may be useful. Some forms of dormancy are overcome by removing the seed coat as in the case of *Trochetiopsis* spp. (Fay, 1992). In some cases, growth

regulators have been used in the culture medium to stimulate seed germination under *in vitro* conditions (Meney and Dixon, 1995). Similarly, seeds of large number of rare orchid species such as *Vanilla walkeriae*, *Cyperipedium reginae* etc. have been asymbiotically germinated under *in vitro* conditions (Agrawal *et al.*, 1992; Faletra *et al.*, 1997).

Although numerous rare and endangered plants have been produced through tissue culture techniques, they all had to be maintained by periodic subcultures. Other disadvantages associated with this method of maintenance, include high cost, the risk of contamination and genetic or phenotypic modification during subculturing. Cryopreservation is a technique which immobilizes metabolic activities of cells, thus suspending ageing and genetic variations (Kartha, 1985), and also has low maintenance costs, providing a cost-effective means for the potential long-term storage of germplasm. The suitability of cryopreservation for secure, long-term storage of rare and endangered species has been carried out in large array of plants especially vegetatively propagated species. This is the only method currently used that could provide ideal conditions for storage of germplasm in liquid nitrogen (LN) at a temperature of -196°C. In recent years, remarkable progress was made in the field of cryopreservation techniques and different plant materials from more than 110 plant species were reported to be adequately stored in LN (http://www.jircas.affrc.go.jp/english/publication/annual/1996/division/okinawa3.html). Consequently, cryopreservation was recognised as a practical, efficient and economic tool for long-term storage of vegetatively propagated plant germplasm including tannia (*Xanthosoma* sp), banana (*Musa* sp), taro (*Colocasia*), yams (*Dioscorea rotundata*), pineapple (*Ananas comosas*), orchids (*Cymbidium*), etc. Recent progress

in cryopreservation has provided potentially valuable cryogenic procedures such as slow freezing, desiccation, encapsulation/dehydration and vitrification. Plant materials mostly used for these procedures are cells, protoplasts, shoot tips, meristems, embryos etc. For successful cryopreservation, it is necessary to avoid lethal intracellular freezing, which occurs during rapid cooling in LN. Thus, cells and meristems have to be sufficiently dehydrated or concentrated before being immersed into LN. Conventional cryopreservation of cultured cells and meristems has been achieved by slow prefreezing to - 40°C in the presence of suitable cryoprotectants (Sugawara and Sakai, 1974; Shimonishi, *et al.*, 1990). Traditional cryopreservation often uses a slow cooling to avoid intracellular ice formation, a common cause of lethal cell damage (Thinh *et al.*, 1999; Lambardi *et al.*, 2000; Touchel *et al.*, 2002). However, the equipment for slow cooling is costly, and the method is not effective for low temperature sensitive species (Pennycooke and Towill, 2000). Recent works has focused on the procedures that would eliminate the need for controlled freezing and enable cells and meristems to be cryopreserved by direct transfer into LN. An approach, called vitrification enables cells and meristems to be cooled to -196°C without ice formation proved to be the most promising (Matsumoto *et al.*, 1994; Takagi *et al.*, 1997; Ishikawa *et al.*, 1996; Hirai and Sakai, 1999; Lambardi *et al.*, 2000; Tsukazaki *et al.*, 2000; Suzuki *et al.*, 2008). Vitrification refers to the physical process by which highly concentrated solution is super cooled to very low temperature and finally solidifies into metastable glass without crystallization. This improved method of vitrification was later used for cropreservation with great success in a number of species such as brome grass (Ishikawa *et al.*, 1996), taro (Takagi *et al.*, 1997), banana (Thinh *et al.*, 1999), sugar beet (Vandenbussche *et al.*, 2000), black

spruce (Touchel *et al.*, 2002), papaya (Wang *et al.*, 2005), *gentiana* (Suzuki *et al.*, 2008), etc.

Apart from the vitrification methods that are applicable mostly to shoot meristms, long-term storage of seeds through cryopreservation is most easy and suitable means to preserve the germplasm variability of rare and endangered plants in term of minimizing the cost and space. The seeds possess the unique ability for coping up the stressful environments. Seeds and spores of a wide variety of species can go dormant for long periods leading to postponement of embryo development until suitable environmental conditions are met for its survivability. Therefore, several laboratories have applied cryopreservation protocols to the seeds of a variety of endangered species. Majority of species currently stored in LN are those with orthodox, or desiccation tolerant seeds. When dried, orthodox seeds generally survive under LN with little or no damage. In some cases, however, seeds may be orthodox, but short-lived. In these cases, they are carefully dried and frozen at - 20°C or in LN. Two examples are the short-lived seeds of *Plantago cordata* and *Salix myricoides* listed as endangered and potentially threatened in Ohio, USA. These have been successfully dried, cryopreserved and banked in LN (Pence, 1999). Other species have desiccation sensitive or 'recalcitrant seeds'. These seeds cannot survive drying, and in the hydrated state they do not survive exposure to LN. Seeds of some large seeded temperate trees, some wetland species, and moist tropics fall in these categories. Excised embryos from these seeds have been largely cryopreserved rather than the whole seeds. Cryopreservation of non-seed tissues, such as immature embryos or *in vitro* cultures offers an alternative approach to be used for the preservation of recalcitrant species (Pence, 1999).

The flowers of *M. spathulata* are pale violet in colour (Fig. 1 A, B); panicles are 10 - 25cm in length; calyx is 6mm in length with acuminate and ovate lobes; corolla is hairy outside and is tubular of 15 - 18mm in length; lateral petals are triangular of 4 x 3mm in size; dorsal petals are oblong of 4.5 x 1.5mm in size; labellum is deeply lobed which is obtuse of 11 - 13 x 4.5 - 5.5mm in size; staminodes are linear to linear lanceolate attached to the filament which is 3mm above the corolla mouth and 6 x 3mm in size; labellum is yellow in colour and obovate (Fig. 1C), the base of which is cuneate with hairy apex and divided into two lobes of 6 - 8 x 2 - 3mm in size; filament is curved of 15 - 17mm in length; anther is 2mm long which consists of semi-lunar wings of 1.5mm length where the crest is quadrate of 0.8mm length; ovary is oblong, glabrous, unilocular with parietal placentation of 2.5 x 0.5mm in size. Flowers last for only 2 - 3 days and produce fruiting capsules with numerous seeds (40 - 50 in number) that burst off at maturity after seed formation.

The flowers of *M. wengeri* are yellow in colour (Fig. 1 D, E); panicles 8 - 23cm long; calyx is pale brown in colour which is tubular, triangular rounded at apex with three-lobes of 6mm length; corolla is yellow in colour, glabrous and tubular at base of 17mm length; lateral petals are triangular, acute, 3 - 5 nerved of 5 x 4mm size; dorsal petals are oblong and curved, 6 x 2.2mm in size; labellum is cordate and deeply lobed which is obtuse and 12 - 14 x 5 - 6mm in size; staminodes are two in number and pale yellow in colour which is linear to linear lanceolate, acuminate, glabrous of 10mm length; filament is 12mm long which is channelled; anthers are 2.5mm long, winged which are longitudinally splitted; ovary is oblong, glabrous, unilocular with parietal placentation of 3.5 x 1mm in size; style which is 25mm long bears funnel shaped stigma (Fig. 1 F). Flowers last for only 2 - 3 days and produce fruiting

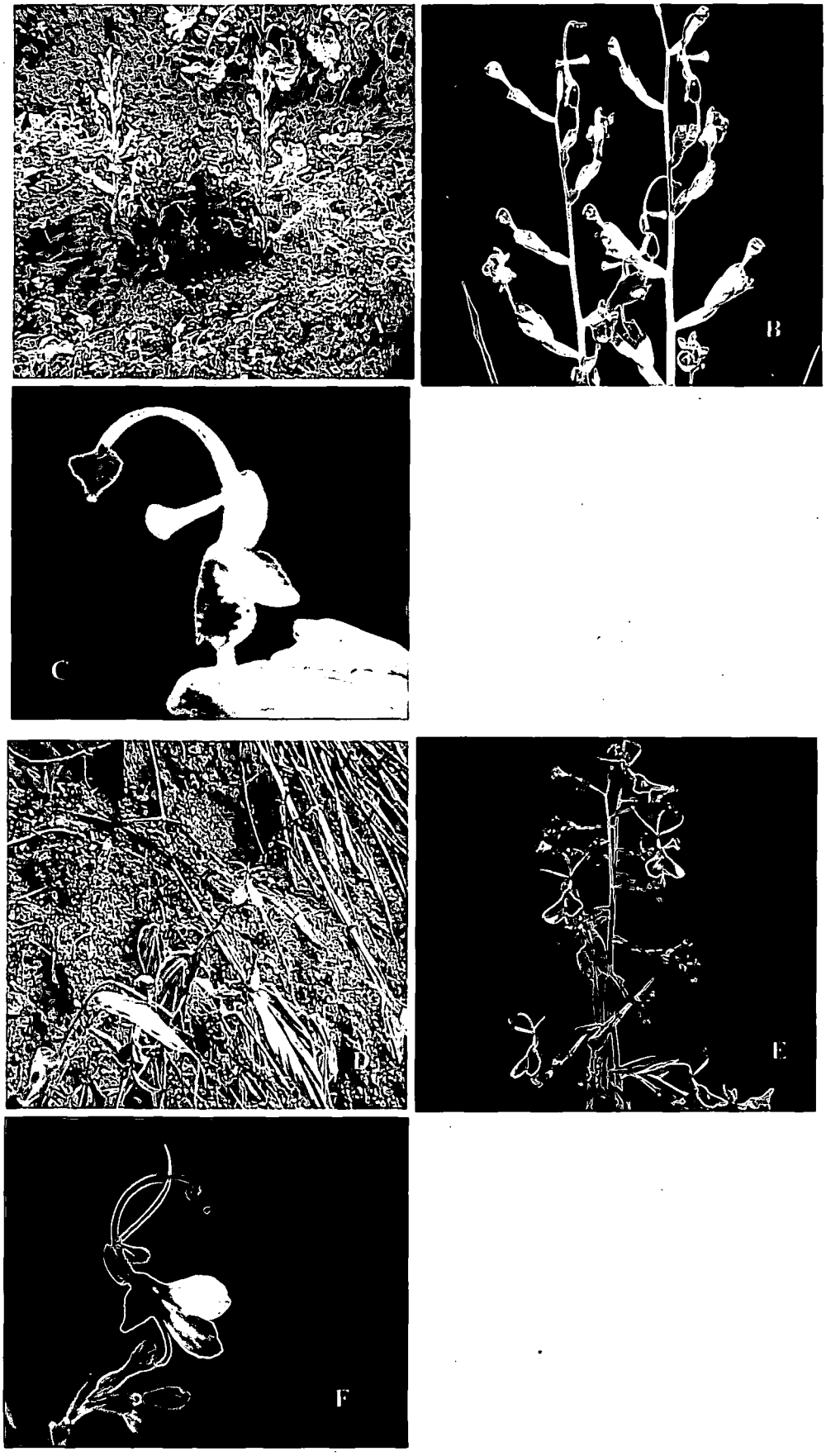


Fig. 1

capsules. Almost all the capsules fall off along with the flower before attaining maturity. However, very few capsules become bigger in size (5 - 7mm) and produce visible seeds (25 - 30 in number). These capsules burst off at maturity after seed formation.

Due to the severe decline in the number of both *M. spathulata* and *M. wengeri* in their natural habitats, experiments were conducted for immediate large-scale propagation and conservation of these species with the following objectives in mind:

1. Micropropagation of *Mantisia spathulata* and *Mantisia wengeri*.
2. Establishment of the hardened plants in experimental garden and natural habitats.
3. Conservation of these species using seed germination and cryopreservation.

These objectives were fulfilled by using the following methods:

1. Survey was conducted in different parts of Mizoram, North-east India for the collection of the two species from their wild habitats. Collected plants were then maintained in the glasshouse.
2. Due to limited availability of plant materials, efforts were given to initiate sufficient aseptic primary cultures for conducting micropropagation experiments. Different explants viz., juvenile leaves, roots and rhizomatous buds after appropriate surface sterilization were inoculated in different nutrient medium viz., Murashige and Skoog (1962), Gamborg's *et al.* (1968), Mitra *et al.* (1976) and Knudson (1946) supplemented with various plant growth regulators for obtaining aseptic shoots.

3. Experiments were conducted to formulate an efficient micropropagation protocol for the *in vitro* multiplication of both the species. The effect of different physio-chemical factors viz., growth regulators, photoperiod, light intensity, temperature, etc. was studied for optimizing the regeneration, growth and development of the plantlets.
4. Various substrata and growth conditions were tested for hardening and acclimatization of *in vitro*-raised plants.
5. As cryptic variations might arise under *in vitro* conditions due to culture stress and mode of regeneration of the plantlets, experiments were conducted to assess the genetic stability of the *in vitro* plantlets with their mother stocks using Random Amplified Polymorphic DNA (RAPD).
6. *In vitro*-raised hardened plantlets were reintroduced under *ex situ* (Experimental Garden of the Department of Botany, NEHU, Shillong) and *in situ* (natural habitats of Lunglei, Mizoram, North-East India).
7. For conserving the genetic diversity of the species, seeds germination under *in vitro* (using different nutrient media supplemented with growth regulators) and *in vivo* (using soil from natural habitats) conditions were carried out.
8. Long-term conservation of the plants was carried out through cryopreservation of the seeds.

CHAPTER II: MICROPROPAGATION OF *MANTISIA SPATHULATA* SCHULT AND *MANTISIA WENGERI* FISCHER: OPTIMIZATION OF PHYSIO-CHEMICAL FACTORS FOR MASS PROPAGATION

INTRODUCTION

When a plant of certain species faces the danger of extinction in its wild habitat, micropropagation remains the only viable alternative for its immediate propagation and recovery. There are many reports where single plant or single population has been conserved through micropropagation. A single plant of *Symonanthus bancroftii* (Solanaceae) that was rediscovered in the vicinity of the Wheetbelt town of Ardath, Western Australia has been conserved by micropropagation (Panaia *et al.*, 2000). Similarly, a number of endangered plants such as *Nepenthes khasiana* and *Nymphaea tetragona* endemic to Meghalaya, *Coptis teeta* endemic to Arunanchal Pradesh, *Mantisia spathulata* and *Mantisia wengeri* endemic to Mizoram, *Dicyospermum ovalifolium* and *Calophyllum apetalum* endemic to Western Ghats have been conserved through micropropagation (Tandon *et al.*, 1990; Rathore *et al.*, 1991; Tandon and Rathore, 1994; Thoyajaksha and Rai, 2001; Nair and Seeni, 2003; Tandon *et al.*, 2007b; Bhowmik *et al.*, 2009). A large number of rare endemic orchids of North-eastern India such as *Cymbidium devonianum, Coelogyne ovalis, Coelogyne nitida, Dendrobium fimbriatum, Dendrobium lituiflorum* etc., have been successfully conserved in recent years (Kumaria and Tandon, 1994; Nongrum *et*

al., 2007; Das *et al.*, 2007; Das *et al.*, 2008). Besides these, numerous herbaceous plants for e.g *Kaempferia galanga* and *Saussurea obvallata*, the 'State flower' of Uttaranchal has also been conserved through micropropagation (Shirin *et al.*, 2000; Joshi and Dhar, 2003). Breeding of zingibers including the species of *Mantisia* is seriously handicapped by poor flowering and seed set. The vegetative multiplication under *in situ* conditions through underground clonal multiplication of rhizomes is usually sluggish. Moreover, the common diseases such as soft rot and yellow rot caused by *Pythium aphanidermatum* and *Fusarium oxysporum* (Dohroo, 1989) and bacterial wilt caused by *Psuedomonas solanaccrum* (Hosoki and Sagawa 1977; De lange *et al.*, 1987) that are widely prevalent in most of the zingibers can also hamper the vegetative multiplication of the species of *Mantisia* in their wild habitat. To overcome the difficulties of conventional propagation, *in vitro* techniques of conservation have been used in a number of cases (Christenson, 1988; Pence *et al.*, 1997).

Plants are micropropagated for various reasons viz. for large-scale propagation, genetic engineering and most important to maintain clones. The use of micropropagation techniques is also useful for supply of annuals and biennials in all seasons, overcoming reproduction barriers caused by sterile male or female plants or barriers in propagation by seeds. It also decreases time of flowering in some plants (Mascarenahas *et al.*, 1982). Typical micropropagation includes several defined steps (Murashige, 1974). The first step is the initiation of a sterile culture of the explant. The second step is the multiplication of shoots. Adventious shoot proliferation is the most frequently used multiplication technology in micropropagation system (Chu, 1992). Maximum rates of multiplication could be obtained by using optimized culture

media and growth conditions. The third stage is the development of roots from the shoots to produce plantlets. The final step is to produce self sufficient plants which usually involve hardening and acclimatization before transferring in the field conditions. Debergh and Maene (1981) introduced another stage, i.e. mother plant selection and preparation. *In vitro* clonal propagation is often difficult but offers an alternative means of mass propagation of plants in limited space, free of microbes and diseases.

In vitro regeneration of plantlets depends on several factors such as age of donor plant (Bhargava and Chandra, 1989; Jain and Chopra, 1988), chemical factors (mainly artificial nutrient medium supplemented with plant hormones, their ratio and type), physical factors [temperature (Prasad *et al.*, 1996), quality and intensity of light (Weis and Jaffe, 1969; Kumar *et al.*, 1999)]. In most of the studies on micropropagation, either apical or axillary buds, nodal segments, rhizome tips, leaf, inflorescences, petiole, embryo, seeds from mature plants, or cotelydonary nodes from aseptically germinated seedlings from zygotic embryos etc. were used as the source of explant.

Micropropagation of species belonging to zingibers has been mostly achieved in nitrogenous rich nutrient medium such as Murashige and Skoogs (MS) medium supplemented with growth regulators mainly 6-benzyl amino purine (BA), Kinetin (KN), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA). The shoot bud multiplication in zingibers have been tried out mostly using meristematic rhizomes in MS medium supplemented with BA and IAA as reported in *Alpinia calcarata* (Agretious *et al.*, 1996). Studies on micropropagation of several species of zingibers have been reported by many workers. Anand and Hariharan

(1997) succeeded in *in vitro* multiplication of *Alpinia galanga* from excised rhizome buds cultured in MS medium supplemented with BA alone or in combination with auxins specially IAA. Prathanturarug *et al.* (2004) accomplished multiple shoot bud formation on MS medium supplemented with BA and NAA in *Zingiber petiolatum*, a rare and endangered plant from southern Thailand. The growth regulator KN played a vital role for bud multiplication in *Amomum subulatum* (Sajina *et al.*, 1997). Plantlets from *in vitro* callus of *Costus speciosus* had been obtained in Schenk and Hildebrandt (SH; 1972) medium supplemented with adenine sulphate, BA, IAA and malt extract by Jain and Chaturvedi (1985). Roy and Pal (1991) reported a rapid multiplication method for *C. speciosus* using shoots derived from zygotic embryos under *in vitro* conditions. Nadgauda *et al.* (1978) regenerated multiple shoots from vegetative buds of *Curcuma longa* in MS medium, supplemented with coconut milk, inositol, KN and BA. Yasuda *et al.* (1988) reported successful callus induction and multiplication in MS medium supplemented with NAA, BA and KN from rhizome explants of *C. aromatica, C. domestica* and *C. zedoaria*. Reghunath and Gopalakrishnan (1991) have standardized *in vitro* techniques for the production of high rate of shoot proliferation using modified MS medium in cardamom. Multiple shoots of *Kaempferia galanga* were regenerated from the rhizome buds in MS medium fortified with BA and NAA (Vincent *et al.*, 1992; Shirin *et al.*, 2000). Microrhizome of *Zingiber officinale* was successfully produced from tissue culture derived shoots by transferring them to liquid MS medium, supplemented with $1mgl^{-1}$ BA, $2mgl^{-1}$ calcium pantothenate, $0.2mgl^{-1}$ GA_3 and $0.5mgl^{-1}$ NAA (Sharma and Singh, 1995). Apart from rhizome and shoot buds, micropropagation has also been accomplished using other explants such as the inflorescence buds of *Alpinia purpurata* (Illg and Faria, 1995).

MATERIALS AND METHODS

Survey and Collection

A survey for the collection of *M. spathulata* and *M. wengeri* was conducted in Lunglei, Mizoram, India during the month of May in 2005, 2006 and 2007. Very few plants (2 - 3) each of *M. wengeri* and *M. spathulata* were collected from their natural habitats and maintained in the glass house of the Plant Biotechnology Laboratory, Botany Department at North Eastern Hill University, Shillong.

Initiation of aseptic primary cultures

Juvenile leaves, roots and rhizomatous buds were selected as a starting material for raising the primary cultures from the limited plant material available. These were washed in running tap water for 1h along with few drops of detergent tween-20 as a measure to remove the dirt from the plant material. The experiments were carried out aseptically in the transfer table of the laminar flow. Before using the transfer table, the laminar flow cabinet was thoroughly scrubbed with cotton soaked in 70% ethanol. The ultra violet (UV) tube light of 30W was switched on for 10 - 15min after which the transfer area was left as such for about 5min with the flow of ultra filtered sterile air to strain out particles as small as 0.3mm providing a sterile atmosphere for work. The velocity of the airflow (27±3m min^{-1}) helped in preventing air-borne contamination. Forceps, needles, surgical blades etc. were sterilized by dipping in alcohol and flamed before use for proper sterilization. Explants such as the leaves (~2 x 2cm), roots (~1cm) and rhizomatous shoot bud (~2 x 1.5cm) were desiccated out from various plant parts and surface disinfected using 0.2% cetramide followed by surface sterilization using various concentrations of mercuric chloride (0.0%, 0.05%, 0.075%, 0.1% and 0.15%) for 2.5, 5.0, 7.5 and 10min for both the

species. The explants were then inoculated in culture tubes containing 15ml of sterilized media viz., Murashige and Skoog (1962; MS), Gamborg's *et al.* (1968; B_5), Mitra *et al.* (1976) and Knudson (1946; KC) supplemented with BA (0.0 - 22.2μM), KN (0.0 - 23.2μM) singly and in combination with NAA (0.0 - 26.8μM). The pH of the media was adjusted to 5.8 prior to autoclaving. The culture tubes were incubated at 25±2°C under 12h photoperiod of 54μmoles $m^{-2}s^{-1}$ light intensity. The medium that supported shoot proliferation was considered as the initiation/induction medium. The same cultural practices were followed for both the species in all the experiments and observations were made after 45 days of culture.

In vitro multiplication of plantlets

Each rhizomatous bud (~2.5 x 2.5mm) obtained from 4 weeks old *in vitro* primary culture was dissected out of the nodal portion. These explants were inoculated in MS medium supplemented with various combinations of growth regulators (BA, KN, NAA, IAA, and IBA) singly and in combination in the range of 0.0 - 20μM to optimize the best concentration for obtaining the maximum of shoot buds and to study the growth and development of the *in vitro* plantlets. Observations were made on the number of shoots formed from each explant and their multiplication capacity was calculated as the bud forming capacity (BFC) using Tandon *et al.* (2007c) formula,

BFC = (average no of buds per explant) x (% of explants forming buds) ÷ 100

NB: Percentage of explants forming buds is the percentage of response of the explants

To study the growth and development of the *in vitro*-raised plantlets, different growth parameters viz., average root number, shoot length, root length and percentage of dry matter were recorded after 45 days of culture. Fresh and dry weights (after

drying in oven at 105°C) of the *in vitro* plantlets were measured on an electronic balance (Sartorius, USA). The average percentage of dry matter was calculated using the formula,

% Dry matter = Average Dry weight ÷ Average Fresh weight x 100

To optimize the physical conditions required for micropropagation of the *Mantisia* species, the explants were cultured in the optimum multiplication medium and subjected to different physical factors such as temperature (4, 8, 16, 24 and 30°C), photoperiod (0, 6, 12, 18 and 24h day), light intensity (0, 13.5, 27.0, 40.5 and 54μmoles $m^{-2}s^{-1}$) and observations were made after 45 days of culture.

Statistical analysis

Ten replicates were maintained for each treatment and the experiment repeated thrice. Statistical analysis was done by Analysis of Variance (ANOVA) at 5% significant level and means compared using Tukeys test (PC version Origin 7.0. NORTHAMPTON, MA, USA).

RESULTS

Survey and Collection

Successful collection of the two species became possible during the flowering season of May, 2006 as the plants could be easily identified with their flowers from the other species of zingibers that were growing in the same habitat. Around 60 - 70 plants of *M. spathulata* (Fig. 1A) and 40 - 50 plants of *M. wengeri* (Fig. 1D) were found growing on the rocky hills along the road sides of two villages of Lunglei, Mizoram viz. Lunglawn and Sethlun respectively at an altitude of around 1100 - 1300m above mean sea level. The habitat of the plants had undergone severe soil

erosions at different places due to heavy rainfall and patches of sliding rocks along with mud and plants were noticed everywhere.

Initiation of aseptic cultures

Around 80 - 90% of the explants dissected out from the leaves of both *M. spathulata* and *M. wengeri* remained green and survived after surface sterilization with lower concentration of sterilant (0.05% $HgCl_2$ for 5min). However, none of the explants was able to regenerate aseptic cultures in any of the media tested. Similarly, 50 - 60% of explants from roots survived the surface sterilization with $HgCl_2$ at 0.1% for 5min but the explants were non-responsive in all the media tested. The explants dissected out from rhizomes showed maximum contamination after surface sterilization with various concentrations of $HgCl_2$ at different time intervals. The exposure of explants to $HgCl_2$ for longer duration resulted in the death of the explants within 2 - 3 days whereas, with the reduction in the exposure time, repeated contamination with fungus resulted. Some explants which escaped fungal contamination were however attacked by bacterial cultures even after several weeks. These bacterial cultures were systematic and were repetitive in all the cultures. As a result after repeated trials, only 20 - 40% of the *in vivo* rhizomatous explants of *M. spathulata* and *M. wengeri* were found to be free of contamination after surface sterilization by 0.1% $HgCl_2$ for 5min. Around 2 - 3 axillary shoot buds (~1 - 2cm) sprouted within 3 - 4 weeks from the nodal portion of rhizomatous bud explants in MS medium incorporated with a combination of 8.8μM BA and 2.7μM NAA for *M. spathulata* (Fig. 2 A, B) and 4.4μM BA and 2.7μM NAA for *M. wengeri* (Fig. 2 C, D). The aseptic cultures obtained were repeatedly multiplied in the same initiation medium to produce sufficient number of primary cultures for subsequent experiments.

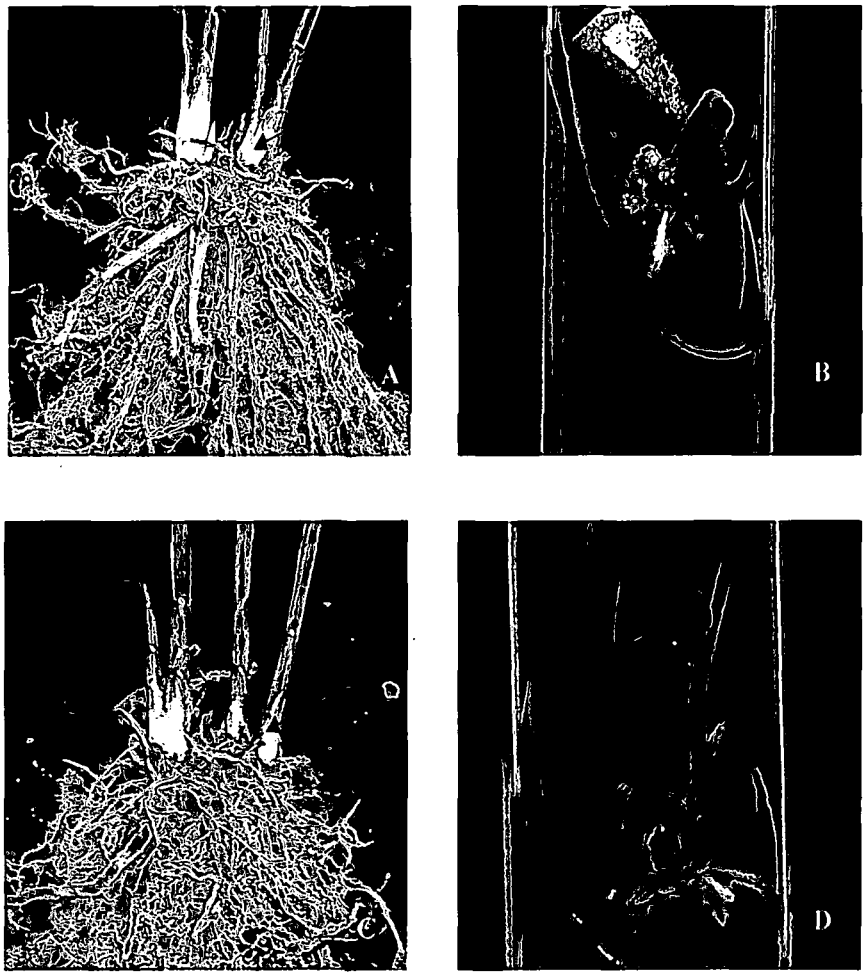


Fig. 2

Hence, further experiments for standardising the protocol for micropropagation of the two species of *Mantisia*, were carried out using rhizomatous explants in MS medium and the effects of different physio-chemical factors were studied on the shoot multiplication, growth and development of the *in vitro* plantlets.

In vitro multiplication of plantlets

A) *Effect of growth regulators singly and in combinations*

Effect of BA

The multiplication of shoot buds in terms of BFC from the explants of *M. spathulata* in MS medium supplemented singly with BA at various concentrations were non significant as compared to the control (Table 2.1). Similarly, the length of shoots and roots were also non significant as compared to the control. However, a maximum of 13.9 roots were obtained in MS medium supplemented with 5μM BA. Around 5.16% dry matter was formed in the *in vitro* plantlets at this concentration after 45 days of culture which was significantly higher than the control.

The multiplication of shoot buds of *M. wengeri* was significantly enhanced with the incorporation of BA in the MS medium with a highest of 5.76 BFC obtained in MS medium supplemented with 10μM BA (Table 2.2). A maximum of 10 roots were formed in MS medium supplemented with 5μM BA. The *in vitro* shoots attained a maximum height of 2.1cm and accumulated 8.36% dry matter within 45 days of culture at this concentration of BA which was significantly higher than the control. However, the lengths of the roots were not significantly increased with the incorporation of BA in the MS medium as compared to the control.

Effect of KN

The multiplication of shoots from the explants of *M. spathulata* in terms of BFC was non significant in MS medium supplemented with various concentrations of KN as compared to the control (Table 2.1). However, the highest number of 15.1 roots was obtained in MS medium supplemented singly with 10μM KN which was significantly higher than the control. The shoots attained a maximum length of 3.26cm within 45 days of culture at 10μM KN in the medium. However, the content of dry matter of the *in vitro* plantlets was significantly inhibited as compared to the control with a highest of only 3.88% dry matter obtained at 10μM KN in the medium. The length of the roots was also non significant in MS medium supplemented with various concentrations of KN as compared to the control.

The multiplication of shoot buds from the explants of *M. wengeri* in terms of BFC was not significantly enhanced in MS medium supplemented singly with various concentrations of KN as compared to the control (Table 2.2). However, a maximum of 11.2 roots of 2.63cm length were obtained in MS medium supplemented with 5μM KN which was significantly higher than the control. The shoots attained a maximum length of 4.2cm at the same concentration. A maximum of 10.83% dry matter was formed in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with 10μM KN.

Effect of NAA

The multiplication of shoot buds from the explants of *M. spathulata* in terms of BFC got significantly inhibited in MS medium supplemented with NAA at 2.5, 10 and 20μM as compared to the control (Table 2.1). However, the numbers of roots from the *in vitro* shoots were significantly enhanced in MS medium supplemented

singly with 5μM NAA. The roots got deformed into clump mass at higher concentrations of NAA (10 and 20μM) in the medium. The shoots attained a maximum length of 1.76cm at 5μM NAA in the MS medium. However, the length of the roots and the percentage of dry matter of the *in vitro* plantlets in MS medium incorporated singly with NAA was non significant as compared to the control.

The multiplication of shoot buds from the explants of *M. wengeri* was significantly inhibited in MS medium supplemented with various concentrations of NAA. However, a highest of only 1.85 BFC was obtained in MS medium supplemented with 10μM NAA (Table 2.2). A significant number of 16.8 roots were also obtained in MS medium at 10μM NAA. The shoots attained a maximum of 3.1cm length with a highest of 2.46cm long roots in MS medium supplemented with 5.0μM NAA which was significantly higher than the control. A maximum of 7.16% dry matter accumulated in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with 10μM NAA.

Effect of IAA

The multiplication of shoot buds from the explants of *M. spathulata* in terms of BFC was not significantly enhanced with the incorporation of IAA in the MS medium (Table 2.1). However, a maximum of only 2.13 BFC and 28.66 roots were obtained at 5μM IAA which was significantly higher than the control. The shoots attained a maximum length of 3.73cm and accumulated a highest of 5.23% dry matter in the *in vitro* plantlets within 45 days of culture at 5μM IAA in the medium. However, the length of roots was not significantly enhanced with the incorporation of IAA in the MS medium.

The multiplication of shoot buds from the explants of *M. wengeri* was enhanced to a maximum of 3.2 BFC in MS medium supplemented with 5μM IAA singly which was significantly higher than the control (Table 2.2). A highest of 9.16 roots and 5.46cm long shoots were obtained in MS medium supplemented with 10μM IAA. The length of the roots attained a maximum of 3.2cm at 20μM IAA which was significantly higher than the control. As compared to the control, the dry matter content of the *in vitro* plantlets got significantly reduced to minimum in MS medium incorporated with various concentrations of IAA. The dry matter got reduced to a minimum of 3.2% at 20μM IAA in the medium within 45 days of culture.

Effect of IBA

The multiplication of shoot buds from the explants of *M. spathulata* were significantly very less with a highest of only 2.52 BFC obtained in MS medium at 5μM IBA as compared to the control (Table 2.1). The multiplication of shoots got completely suppressed at higher concentrations with a minimum of only 0.63 BFC at 20μM IBA in the medium. However, a maximum of 18.8 roots were formed in the *in vitro* shoots in MS medium supplemented with 20μM IBA. The shoots as well as the roots attained a maximum height of 3cm and 2.66cm respectively in MS medium supplemented with 10μM IBA which was significantly higher than the control. As compared to the control, there was slight increase in the dry matter content (4.45%) of the *in vitro* plantlets in the MS medium at 10μM IBA.

As compared to the control, the multiplication of shoot buds from the explants of *M. wengeri* in MS medium supplemented with IBA was non significant and got completely inhibited (Table 2.2). However, a maximum of 9.26 roots were formed in the *in vitro* shoots in MS medium supplemented with 10μM IBA. The shoots attained

Table 2.1: Effect of growth regulators (BA, KN, NAA, IAA and IBA) incorporated singly in MS medium on shoot multiplication, growth and development of *M. spathulata*

Growth regulator	Response* (%)	BFC*	Root no.*	Shoot length (cm) *	Root length (cm) *	Dry Matter* (%)
0.0	93.3±6.6^{a}	1.5±0.3^{a}	2.9±0.4^{a}	2.6±0.1^{a}	1.7±0.2^{a}	4.3±0.1^{a}
BA						
2.5	86.6±6.6	2.3±0.76	8.6±2.86^{abc}	1.86±0.43	1.34±0.25	3.9±0.65^{ab}
5.0	93.3±6.6	3.4±0.63	13.9±2.37^{bc}	2.16±0.33	1.76±0.28	5.16±0.34^{ab}
10.0	80.0±0.0	2.1±0.23	7.30±0.80^{a}	3.13±0.12	1.12±0.20	3.70±0.49^{ab}
20.0	66.6±6.6	1.0±0.10	3.0±0.28^{a}	1.86±0.46	0.87±0.21	2.96±0.17^{a}
	NS	NS		NS	NS	
KN						
2.5	53.3±6.6^{b}	0.71±0.28	4.46±0.43^{a}	1.80±0.26^{a}	0.96±0.12	2.66±0.26^{b}
5.0	60.0±0.0^{b}	0.84±0.18	5.53±0.17^{a}	2.76±0.14^{ab}	1.18±0.16	3.15±0.02^{b}
10.0	66.6±6.6^{a}	1.68±0.34	15.10±3.0^{b}	3.26±0.23^{ab}	1.96±0.53	3.88±0.96^{ac}
20.0	60.0±0.0^{b}	1.04±0.21	4.10±0.43^{a}	1.60±0.32^{a}	1.43±0.46	3.24±0.04^{bc}
		NS			NS	
NAA						
2.5	60.0±0.0^{b}	0.84±0.06^{ac}	40.13±1.18^{b}	1.12±0.24^{b}	1.26±0.145	4.66±0.2
5.0	73.3±6.6^{a}	1.62±0.35^{ac}	49.93±3.95^{b}	1.76±0.18^{bc}	1.83±0.145	5.05±0.19
10.0	53.3±6.6^{b}	0.40±0.08^{a}	DCR	0.68±0.12^{b}	-	4.40±0.21
20.0	46.6±6.6^{b}	0.34±0.07^{b}	DCR	0.57±0.07^{b}	-	4.10±0.23
					NS	NS
IAA						
2.5	73.3±6.6^{a}	1.73±0.26	22.53±1.72^{b}	3.5±0.17^{abc}	1.5±0.2	5.12±0.34^{ab}
5.0	73.3±6.6^{a}	2.13±0.59	28.66±0.97^{bc}	3.73±0.27^{bc}	1.8±0.15	5.23±0.035^{ab}
10.0	53.3±6.6^{b}	1.07±0.28	15.36±1.41^{b}	2.66±0.26^{abc}	2.0±0.11	4.38±0.13^{ab}
20.0	46.6±6.6^{b}	0.42±0.08	11.56±0.89^{b}	2.50±0.28 ^{a}	1.3±0.14	3.97±0.23^{a}
		NS			NS	
IBA						
2.5	80.0±0.0^{a}	1.49±0.05^{a}	6.10±0.72^{a}	1.9±0.26^{a}	1.80±0.1^{a}	3.77±0.34^{ab}
5.0	86.6±6.6^{a}	2.52±0.3^{a}	11.8±0.93^{b}	2.3±0.25^{ac}	2.36±0.29^{abc}	3.99±0.12^{ab}
10.0	93.3±6.6^{a}	1.68±0.26^{a}	15.3±1.0^{bc}	3.0±0.20^{ac}	2.66±0.14^{bc}	4.45±0.08^{ab}
20.0	66.6±6.6^{b}	0.63±0.08^{b}	18.8±0.95^{bc}	1.2±0.11^{b}	1.20±0.12^{a}	3.53±0.63^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE); (-): No response; NS: Non-significant; DCR: Deformed clump roots:
ANOVA at 5% level of significance shows that all the growth parameters are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p= 0.05)
Data recorded after 45 days of culture

Table 2.2: Effect of growth regulators (BA, KN, NAA, IAA and IBA) incorporated singly in MS medium on shoot multiplication, growth and development of *M. wengeri*

Growth regulator	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0	93.3±6.6	1.53±0.17^{a}	4.20±0.61^{a}	3.3±0.36^{a}	1.0±0.15	4.96±0.14^{a}
BA						
2.5	86.6±6.6	3.42±0.32^{b}	4.66±1.2^{abc}	1.51±0.29^{a}	1.30±0.28	7.53±0.76^{bc}
5.0	93.3±6.6	5.14±0.53^{bc}	10.0±2.0^{bc}	2.10±0.23^{a}	1.46±0.20	8.36±0.31^{bc}
10.0	93.3±6.6	5.76±0.52^{bc}	3.70±0.45^{a}	1.13±0.06^{a}	0.65±0.07	5.33±0.43^{a}
20.0	80.0±0.0	3.84±0.12^{b}	1.66±1.2^{a}	1.06±0.17^{a}	0.64±0.08	3.93±0.2^{a}
	NS				NS	
KN						
2.5	86.6±6.6	1.73±0.33	9.60±0.5^{bc}	2.6±0.23^{a}	1.83±0.17^{abc}	9.96±1.13^{bc}
5.0	93.3±6.6	2.26±0.86	11.2±0.8^{bc}	4.2±0.23^{ab}	2.63±0.21^{bc}	10.10±0.77^{bc}
10.0	86.6±6.6	1.66±0.46	8.13±0.17^{b}	3.5±0.20^{ab}	2.33±0.17^{bc}	10.83±0.95^{bc}
20.0	86.6±6.6	1.21±0.13	6.06±0.46^{a}	3.3±0.32^{ab}	1.20±0.23^{a}	9.36±0.35^{bc}
	NS	NS				
NAA						
2.5	73.3±6.6^{a}	1.41±0.17^{ac}	13.6±1.8^{bc}	2.8±0.23^{a}	2.26±0.12^{bc}	5.26±0.2^{a}
5.0	73.3±6.6^{a}	1.48±0.23^{ac}	15.3±1.21^{bc}	3.1±0.44^{a}	2.46±0.20^{bc}	6.90±0.51^{bc}
10.0	66.6±6.6^{b}	1.85±0.15^{ac}	16.8±2.25^{bc}	1.8±0.30^{a}	1.36±0.3^{a}	7.16±0.23^{bc}
20.0	46.6±6.6^{b}	0.22±0.08^{b}	5.0±1.28^{a}	1.0±0.37^{b}	0.65±0.17^{b}	2.83±0.14^{b}
IAA						
2.5	60.0±0.0^{b}	1.48±0.10^{a}	7.6±0.61^{bc}	3.86±0.17^{a}	1.2±0.11^{a}	4.13±0.24^{a}
5.0	86.6±6.6^{a}	3.20±0.42^{bc}	8.2±0.43^{bc}	5.33±0.08^{bc}	1.5±0.11^{a}	4.86±0.17^{a}
10.0	73.3±6.6^{a}	2.69±0.53^{abc}	9.16±0.26^{bc}	5.46±0.24^{bc}	2.9±0.2^{bc}	4.46±0.35^{a}
20.0	66.6±6.6^{b}	1.24±0.28^{a}	4.16±0.23^{a}	2.50±0.28^{a}	3.2±0.2^{bc}	3.20±0.30^{b}
IBA						
2.5	73.3±6.6	1.76±0.18	6.26±0.56^{a}	0.83±0.14^{b}	1.30±0.17^{a}	4.8±0.37^{a}
5.0	80.0±0.0	0.89±0.26	7.60±0.83^{bc}	1.40±0.17^{b}	3.13±0.17^{b}	5.6±0.23^{ab}
10.0	80.0±6.6	0.87±0.26	9.26±0.63^{bc}	2.76±0.26^{a}	4.33±0.37^{bc}	6.2±0.23^{ab}
20.0	66.6±6.6	0.79±0.32	5.53±0.31^{a}	0.95±0.25^{b}	2.10±0.23^{b}	4.4±0.40^{a}
	NS	NS				

'*' indicates mean average values of three repeated experiments with standard error (±SE); (-): No response; NS: Non-significant

ANOVA at 5% level of significance shows that all the parameters of are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$);

Data recorded after 45 days of culture

a maximum length of 3.3cm in the control which was significantly higher than IBA supplemented MS medium. The roots attained a maximum length of 4.33cm and the plantlets accumulated a highest of 6.2% dry matter within 45 days of culture in MS medium supplemented with 10μM IBA which was significantly higher than the control.

Effect of BA + NAA

The multiplication of shoot buds from the explants of *M. spathulata* was significantly enhanced in MS medium supplemented with BA and NAA at various concentrations as compared to control (Table 2.3). A maximum of 6.1 BFC was recorded from the explants in MS medium supplemented with a combination of 10μM BA and 2.5μM NAA. As compared to the other growth regulators used at varying concentrations, this particular combination of BA and NAA promoted the highest shoot bud multiplication in terms of BFC and therefore was considered to be the optimum concentration for highest shoot proliferation and plantlets development of *M. spathulata* (Fig. 3 A - D). As compared to the control the BFC got significantly inhibited at higher concentrations of BA and NAA with a minimum of only 0.24 BFC at 2.5μM BA in combination with 20μM NAA in the MS medium. There was a significant increase in the number of roots (19.93) from the *in vitro shoots* in MS medium supplemented with a combination of 10μM BA and 2.5μM NAA. The roots got deformed into clump mass at higher concentrations of BA (5μM, 10μM, 20μM) and NAA (10μM) in combination within 45 days of culture. The shoots attained a maximum length of 3.53cm in MS medium supplemented with a combination of 10μM BA and 2.5μM NAA. Similarly, the roots attained a maximum length of 3cm at 5μM BA and 2.5μM NAA in combination which was significantly higher than the

control. The *in vitro* plantlets accumulated significantly a highest of 7.76% dry matter within 45 days of culture in MS medium supplemented with a combination of 10μM BA and 2.5μM NAA.

The shoot multiplication from the explants of *M. wengeri* was significantly enhanced at varying concentrations of BA and NAA in combinations in MS medium (Table 2.4). A maximum of 7.82 BFC was obtained from the explants in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA which was significantly higher than the BFC obtained in control and at other concentrations of BA and NAA in combinations. As compared to the other growth regulators used at varying concentrations, this particular combination of BA and NAA promoted the highest shoot bud multiplication in terms of BFC and was therefore considered to be the optimum concentration for highest shoot proliferation and plantlets development of *M. wengeri* (Fig. 3 E - H). However, combination of BA at higher concentrations of NAA (20μM) showed inhibition of shoot multiplication in the medium. A highest of 20.9 roots was obtained from the *in vitro* shoots in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA in the MS medium. However, length of the shoots got significantly elongated to a highest of 5.9cm in MS medium supplemented with a combination of 2.5μM BA and 10μM NAA. The roots attained significantly a highest length of 3.16cm in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA as compared to the control. A significant amount of dry matter (9.83%) was accumulated in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with a combination of 2.5μM BA and 10μM NAA.

Table 2.3: Effect of BA and NAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. spathulata*

BA+NAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	1.42±0.18^{a}	3.0±0.4^{a}	2.6±0.1^{a}	1.60±0.15^{a}	4.2±0.17^{a}
2.5+2.5	93.3±6.6^{a}	3.16±0.49^{a}	7.8±0.75^{a}	2.8±0.51^{ac}	2.73±0.26^{ab}	4.7±0.11^{a}
5.0+2.5	93.3±6.6^{a}	4.25±0.64^{b}	11.0±0.90^{b}	3.2±0.50^{ac}	3.0±0.20^{ab}	6.46±0.14^{bc}
10.0+2.5	93.3±6.6^{a}	6.10±0.55^{bc}	19.93±3.19^{bc}	3.53±0.29^{ac}	2.8±0.15^{ab}	7.76±0.83^{bc}
20.0+2.5	93.3±6.6^{a}	2.60±0.51^{a}	18.40±0.87^{bc}	2.40±0.23^{ac}	1.96±0.53^{ab}	5.06±0.43^{a}
2.5+5.0	66.6±6.6^{b}	1.33±0.39^{a}	11.73±1.09^{b}	1.13±0.20^{b}	1.03±0.35^{a}	3.26±0.20^{a}
5.0+5.0	73.3±6.6^{a}	2.25±0.40^{a}	15.83±1.85^{bc}	1.14±0.18^{a}	1.06±0.31^{a}	5.26±0.14^{a}
10.0+5.0	60.0±0.0^{b}	2.28±0.30^{a}	17.56±2.01^{bc}	2.43±0.21^{ac}	2.16±0.32^{ab}	5.70±0.05^{a}
20.0+5.0	53.3±6.6^{b}	1.32±0.13^{a}	12.90±1.86^{bc}	3.16±0.34^{ac}	2.53±0.14^{ab}	5.56±0.14^{a}
2.5+10.0	60.0±0.0^{b}	0.85±0.12^{a}	6.10±1.7^{a}	1.36±0.27^{a}	0.86±0.32^{a}	4.1±0.11^{a}
5.0+10.0	46.6±6.6^{b}	1.32±0.13^{a}	CM	0.8±0.05^{b}	CM	5.0±0.51^{a}
10.0+10.0	40.0±0.0^{b}	0.32±0.0^{a}	CM	0.6±0.05^{b}	CM	4.2±0.55^{a}
20.0+10.0	40.0±0.0^{b}	0.25±0.04^{a}	CM	0.43±0.06^{b}	CM	3.1±0.30^{a}
2.5+20.0	40.0±0.0^{b}	0.24±0.02^{a}	CM	0.26±0.08^{b}	CM	2.2±0.40^{b}
5.0+20.0	-					
10.0+20.0	-					
20.0+20.0	-					

'*' indicates mean average values of three repeated experiments with standard error (±SE); CM: clump; (-): No response

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)

Data recorded after 45 days of culture

Table 2.4: Effect of BA and NAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. wengeri*

BA+NAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	2.09±0.44^{a}	4.3±1.05^{a}	2.73±0.29^{a}	1.03±0.14^{a}	4.9±0.14^{a}
2.5+2.5	73.3±6.6^{a}	2.04±0.37^{a}	13.8±1.82^{bc}	2.50±0.26^{a}	2.33±0.17^{abc}	5.9±0.43^{abc}
5.0+2.5	93.3±6.6^{a}	7.82±0.73^{bc}	20.9±1.65^{bc}	2.86±0.43^{a}	3.16±0.29^{bc}	8.2±0.25^{abc}
10.0+2.5	93.3±6.6^{a}	5.73±0.58^{b}	13.5±0.89^{bc}	1.33±0.36^{a}	2.2±0.52^{abc}	7.3±0.1^{abc}
20.0+2.5	93.3±6.6^{a}	3.36±0.50^{a}	8.03±1.33^{a}	1.08±0.35^{b}	1.4±0.23^{abc}	5.1±0.20^{a}
2.5+5.0	60.0±11.5^{b}	1.32±0.56^{a}	6.86±1.43^{a}	0.95±0.34^{b}	1.1±0.06^{abc}	2.62±0.39^{a}
5.0+5.0	73.3±6.6^{a}	2.45±0.41^{a}	7.93±0.75^{a}	1.33±0.37^{a}	1.4±0.14^{abc}	4.8±0.28^{a}
10.0+5.0	86.6±6.6^{a}	2.46±0.60^{a}	10.9±0.86^{a}	1.43±0.29^{a}	1.6±0.49^{abc}	5.3±0.23^{a}
20.0+5.0	93.3±6.6^{a}	3.93±0.83^{a}	4.73±0.73^{a}	1.16±0.16^{b}	2.56±0.14^{abc}	8.0±0.49^{abc}
2.5+10.0	66.6±6.6^{b}	1.25±0.43^{a}	18.9±2.46^{bc}	5.90±0.55^{bc}	2.10±0.17^{abc}	9.83±1.2^{bc}
5.0+10.0	80.0±11.5^{a}	2.02±0.63^{a}	14.8±2.45^{bc}	3.23±0.33^{a}	2.56±0.14^{abc}	8.70±0.43^{abc}
10.0+10.0	80.0±11.5^{a}	2.30±0.64^{a}	12.0±1.5^{a}	2.53±0.31^{a}	2.33±0.20^{abc}	6.86±0.34^{abc}
20.0+10.0	73.3±13.3^{a}	1.49±0.66^{a}	7.86±0.78^{a}	1.86±0.23^{a}	2.16±0.35^{abc}	6.22±0.6^{abc}
2.5+20.0	20.0±0.0^{b}	0.1±0.08^{a}	2.73±1.39^{a}	0.81±0.54^{b}	0.76±0.15 ^{a}	1.66±0.33^{a}
5.0+20.0	20.0±0.0^{b}	0.1±0.07^{a}	3.16±2.32^{a}	1.06±0.37^{a}	1.06±0.37^{abc}	2.83±0.14^{a}
10.0+20.0	26.6±6.6^{b}	0.5±0.46^{a}	3.6±1.97^{a}	1.50±0.15^{b}	0.3±0.19^{a}	3.6±0.29^{a}
20.0+20.0	26.6±6.6^{b}	0.3±0.26^{a}	0.66±0.16^{a}	0.30±0.19^{b}	0.15±0.15^{a}	2.7±1.86^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE) ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 45 days of culture

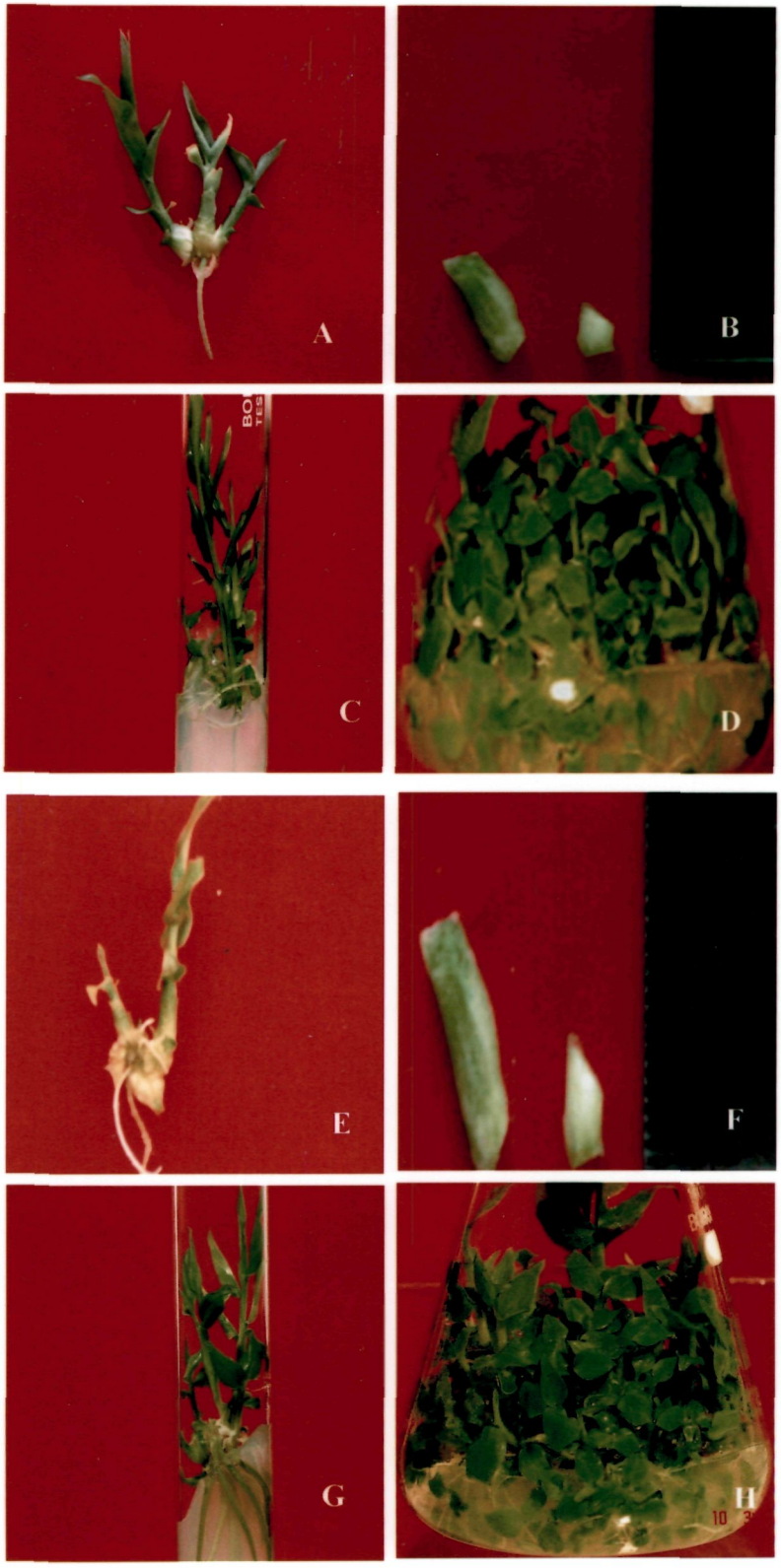


Fig. 3

Effect of KN + NAA

The multiplication of shoot buds from the explants of *M. spathulata* in terms of BFC was significantly increased at lower concentrations of KN and NAA in combinations as compared to the control (Table 2.5). A maximum of 3.2 BFC was recorded from the explants in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA. However, the shoot multiplication was recorded to get significantly inhibited beyond this concentration of KN and NAA with a minimum of only 0.21 BFC in equal concentrations of KN and NAA at 10μM in the medium. A maximum of 21.66 roots were formed from the *in vitro* shoots in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA which was significantly higher than the control as well as the other concentrations of KN and NAA used in combinations. The shoots attained a maximum length of 5.4cm in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA. However, the roots attained a maximum length of 3.83cm in MS medium supplemented with a combination of 10μM BA and 2.5μM NAA. A significant amount of dry matter (9.26%) were formed in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with a combination of 5μM BA and 2.5μM NAA.

The multiplication of shoot buds from the explants of *M. wengeri* was recorded to get significantly suppressed at various concentrations of KN and NAA in combinations in MS medium as compared to the control. The shoot multiplication from the explants got completely inhibited in MS medium incorporated with a combination of KN (2.5μM, 5μM, 10μM, 20μM) and 20μM NAA (Table 2.6). However, a highest of only 2.52 BFC was recorded in MS medium supplemented with a combination of 5μM KN and 2.5μM NAA. A maximum of 18.93 roots were

Table 2.5: Effect of KN and NAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. spathulata*

KN+NAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	1.42±0.18^{a}	3.0±0.4^{a}	2.60±0.1^{a}	1.60±0.15^{a}	4.2±0.17^{a}
2.5+2.5	73.3±6.6^{a}	1.62±0.17^{a}	9.4±0.61^{a}	2.07±0.12^{a}	1.82±0.12^{a}	6.2±0.13^{b}
5.0+2.5	93.3±6.6^{a}	3.2±0.61^{bc}	21.66±1.83^{bc}	5.40±0.30^{bc}	3.23±0.14^{bc}	9.26±0.24^{bc}
10.0+2.5	86.6±6.6^{a}	1.84±0.28^{a}	19.13±1.87^{bc}	4.16±0.37^{b}	3.83±0.2^{bc}	6.66±0.52^{b}
20.0+2.5	86.6±6.6^{a}	1.12±0.16^{a}	12.70±1.30^{b}	2.46±0.08^{a}	2.13±0.06^{a}	5.20±0.15^{a}
2.5+5.0	66.6±6.6^{b}	0.82±0.07^{a}	8.46±1.16^{a}	0.92±0.13^{b}	0.93±0.03^{a}	4.76±0.17^{a}
5.0+5.0	86.6±6.6^{a}	1.65±0.19^{a}	14.96±1.0^{b}	1.67±0.08 ^{a}	2.66±0.18^{b}	6.66±0.14^{b}
10.0+5.0	80.0±0.0^{a}	0.68±0.08^{a}	16.3±1.53^{bc}	4.56±0.20^{bc}	2.20±0.23^{a}	8.20±0.11^{bc}
20.0+5.0	73.3±6.6^{a}	0.29±0.09^{a}	14.0±1.39^{b}	3.03±0.88 ^{a}	0.66±0.08^{b}	7.30±0.20^{b}
2.5+10.0	40.0±0.0^{b}	0.25±0.04^{b}	8.0±0.82^{b}	0.72±0.40^{b}	0.44±0.25^{b}	4.53±0.17^{a}
5.0+10.0	53.3±6.6^{b}	0.48±0.11^{a}	6.76±0.56^{b}	1.53±0.17^{b}	0.5±0.28^{b}	5.30±0.23^{a}
10.0+10.0	53.3±6.6^{b}	0.21±0.07^{b}	5.25±0.28^{b}	1.01±0.10^{b}	0.13±0.07^{b}	3.90±0.23^{a}
20.0+10.0	-					
2.5+20.0	-					
5.0+20.0	-					
10.0+20.0	-					
20.0+20.0	-					

'*' indicates mean average values of three repeated experiments with standard error (±SE); (-): No response

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)

Data recorded after 45 days of culture

Table 2.6: Effect of KN and NAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. wengeri*

KN+NAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	2.09±0.44^{a}	4.30±1.05^{a}	2.73±0.29^{a}	1.03±0.14^{a}	4.90±0.14^{a}
2.5+2.5	66.6±6.6^{b}	1.72±0.35^{ab}	10.86±0.69^{b}	1.73±0.12^{a}	1.83±0.23^{abc}	11.23±0.63^{abc}
5.0+2.5	86.6±6.6^{a}	2.52±0.34^{ab}	16.16±2.18^{bc}	2.26±0.26^{a}	2.40±0.65^{abc}	12.3±1.12^{abc}
10.0+2.5	93.3±6.6^{a}	2.08±0.34^{ab}	18.0±1.56^{bc}	3.90±0.37^{abc}	2.26±0.29^{abc}	14.16±2.61^{bc}
20.0+2.5	73.3±6.6^{a}	1.30±0.3^{ab}	13.0±0.91^{bc}	2.13±0.29^{a}	1.53±0.14^{abc}	10.34±0.95^{abc}
2.5+5.0	73.3±6.6^{a}	1.28±0.24^{ab}	13.73±1.0^{bc}	2.63±0.35^{a}	2.06±0.20^{abc}	9.04±1.38^{abc}
5.0+5.0	93.3±6.6^{a}	2.06±0.29^{ab}	18.5±1.1^{bc}	3.50±0.26^{a}	2.36±0.2^{abc}	9.38±0.42^{abc}
10.0+5.0	93.3±6.6^{a}	2.35±0.44^{ab}	15.53±0.88^{bc}	4.73±0.14^{bc}	2.86±0.4^{bc}	10.42±1.12^{abc}
20.0+5.0	93.3±6.6^{a}	1.98±0.20^{ab}	14.6±1.82^{bc}	1.90±0.11^{a}	2.13±0.08^{abc}	8.65±0.36^{a}
2.5+10.0	60.0±0.0^{b}	0.56±0.10^{a}	16.06±0.59^{bc}	2.80±0.17^{a}	1.26±0.12^{a}	7.58±0.60^{a}
5.0+10.0	73.3±6.6^{a}	1.60±0.60^{ab}	18.93±0.63^{bc}	2.53±0.08^{a}	1.46±0.18^{abc}	9.92±0.56^{abc}
10.0+10.0	86.6±6.6^{a}	1.12±0.24^{ab}	6.80±0.75^{a}	2.03±0.14^{a}	1.10±0.2^{a}	16.6±3.91^{bc}
20.0+10.0	53.3±6.6^{b}	0.48±0.12^{a}	5.60±0.6^{a}	1.03±0.08^{b}	0.70±0.15^{a}	10.0±1.10^{abc}
2.5+20.0	-					
5.0+20.0	-					
10.0+20.0	-					
20.0+20.0	-					

'*' indicates mean average values of three repeated experiments with standard error (±SE); (-): No response

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)

Data recorded after 45 days of culture

formed from the *in vitro* shoots in MS medium supplemented with a combination of 5μM KN and 10μM NAA. The shoots as well as the roots attained a highest length of 4.73cm and 2.86cm respectively in MS medium supplemented with a combination of 10μM KN and 5μM NAA which was significantly higher than the control. The *in vitro* plantlets accumulated a maximum of 16.6% dry matter within 45 days of culture in MS medium supplemented with equal concentrations of KN and NAA at 10μM in the medium.

Effect of BA + IAA

The multiplication of shoots from the explants of *M. spathulata* in MS medium incorporated with varying concentrations of BA and IAA in combination was recorded to get slightly increased as compared to the control (Table 2.7). However, a maximum of only 3.96 BFC was recorded in MS medium supplemented with a combination of 10μM BA and 5μM IAA which was significantly higher than the control. A sufficient number of significantly healthy roots (37.13) were formed from the *in vitro* shoots in MS medium supplemented with equal concentrations of BA and IAA at 5μM in combination. However, the roots were deformed into clump mass in MS medium incorporated with a combination of BA (5μM, 10μM, 20μM) and 20μM IAA. The length of the shoots got significantly enhanced to a highest of 3.83cm length in MS medium supplemented with a combination of 5μM BA and 2.5μM IAA. Similarly, a maximum of 3cm long roots and 6.26% dry matter of the *in vitro* plantlets were obtained within 45 days of culture in MS medium supplemented with equal concentration of BA and IAA at 5μM in combination.

The multiplication of shoot buds from the explants of *M. wengeri* was recorded to get significantly enhanced at various concentrations of BA and IAA in

Table 2.7: Effect of BA and IAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. spathulata*

BA+IAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	1.42±0.18^{a}	3.0±0.4^{a}	2.60±0.1^{a}	1.6±0.15^{a}	4.2±0.17^{a}
2.5+2.5	86.6±6.6^{a}	2.44±0.26^{a}	17.65±1.3^{b}	2.73±0.12^{a}	2.5±0.11^{bc}	5.0±0.20^{b}
5.0+2.5	86.6±6.6^{a}	3.17±0.25^{bc}	26.6±1.33^{b}	3.83±0.12^{bc}	2.71±0.15^{bc}	5.4±0.11^{b}
10.0+2.5	86.6±6.6^{a}	2.82±0.34^{abc}	23.3±1.15^{b}	1.86±0.08^{b}	2.81±0.10^{bc}	5.0±0.05^{b}
20.0+2.5	86.6±6.6^{a}	2.44±0.28^{a}	11.1±0.81^{b}	1.80±0.05^{b}	1.16±0.13^{a}	5.0±0.1^{b}
2.5+5.0	93.3±6.6^{a}	3.46±0.22^{bc}	29.7±1.43^{b}	1.50±0.11^{b}	1.53±0.08^{a}	5.56±0.12^{b}
5.0+5.0	93.3±6.6^{a}	3.66±0.28^{bc}	37.13±1.06^{c}	2.33±0.17^{a}	3.00±0.1^{bc}	6.26±0.08^{bc}
10.0+5.0	93.3±6.6^{a}	3.96±0.38^{bc}	11.43±1.2^{b}	1.16±0.14^{b}	0.95±0.08^{b}	6.06±0.08^{bc}
20.0+5.0	73.3±6.6^{a}	2.61±0.09^{abc}	8.20±0.80^{a}	0.85±0.08^{b}	0.75±0.20^{a}	5.00±0.1^{b}
2.5+10.0	86.6±6.6^{a}	2.10±0.19^{a}	10.95±1.52^{b}	0.62±0.07^{b}	0.90±0.23^{a}	3.63±0.14^{a}
5.0+10.0	73.3±6.6^{a}	2.90±0.5^{bc}	12.15±1.24^{b}	0.90±0.17^{b}	1.66±0.08^{a}	4.96±0.066^{b}
10.0+10.0	60.0±0.0^{b}	2.07±0.35^{a}	22.85±2.94^{b}	2.95±0.37^{a}	1.53±0.27^{a}	5.03±0.088^{b}
20.0+10.0	60.0±0.0^{b}	1.38±0.10^{a}	12.3±1.15^{b}	1.66±0.08^{b}	0.96±0.08^{a}	4.33±0.23^{a}
2.5+20.0	66.6±6.6^{b}	0.45±0.05^{a}	16.55±0.83^{b}	0.65±0.03^{b}	1.10±0.11^{a}	4.53±0.088^{a}
5.0+20.0	66.6± 6.6^{b}	1.02±0.35^{a}	DCR	1.46±0.06^{b}	-	3.20±0.1^{b}
10.0+20.0	86.6±0.0^{a}	0.41±0.11^{a}	DCR	0.45±0.03^{b}	-	3.00±0.057^{b}
20.0+20.0	60.0± 6.6^{b}	0.37±0.07^{a}	DCR	0.30±0.06^{b}	-	2.16±0.07^{b}

'*' indicates mean average values of three repeated experiments with standard error (±SE); DCR: Deformed clump roots; (-): No response

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)

Data recorded after 45 days of culture

Table 2.8: Effect of BA and IAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. wengeri*

BA+IAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	2.09±0.44^{a}	4.30±1.05^{a}	2.73±0.29^{a}	1.03±0.14^{a}	4.90±0.14^{a}
2.5+2.5	46.6±6.6^{b}	0.88±0.28^{a}	1.46±0.13^{a}	0.38±0.10^{a}	0.48±0.18^{a}	1.43±0.32^{b}
5.0+2.5	60.0±11.5^{b}	1.72±0.53^{b}	1.86±0.46^{a}	0.76±0.26^{a}	0.70±0.34^{a}	1.86±0.29^{b}
10.0+2.5	80.0±11.5^{a}	4.05±0.76^{bc}	3.20±0.50^{a}	1.15±0.18^{a}	1.75±0.18^{a}	8.03±0.17^{ac}
20.0+2.5	60.0±0.0^{b}	1.48±0.14^{a}	1.66±1.12^{a}	0.64±0.05^{b}	0.32±0.18^{a}	7.63±0.08^{ac}
2.5+5.0	86.6±6.6^{a}	6.22±0.79^{bc}	13.26±1.5^{bc}	2.68±0.24^{b}	4.53±.24^{bc}	7.93±0.17^{ac}
5.0+5.0	86.6±6.6^{a}	4.56±0.53^{bc}	9.60±1.4^{abc}	2.82±0.25^{b}	3.13±0.20^{b}	7.70±0.26^{ac}
10.0+5.0	83.3±6.7^{a}	4.13±0.37^{bc}	6.13±0.98^{b}	2.13±0.29^{b}	1.25±0.24^{a}	4.56±0.32^{ac}
20.0+5.0	66.6±6.6^{b}	1.77±0.24^{a}	4.46±0.29^{a}	1.63±0.24^{b}	0.59±0.09^{a}	4.03±0.37^{a}
2.5+10.0	63.3±3.3^{b}	3.65±0.43^{b}	4.00±0.69^{a}	2.60±0.15^{b}	1.80±0.17^{a}	8.93±0.32^{ac}
5.0+10.0	73.3±6.6^{a}	3.20±0.42^{b}	4.16±0.53^{a}	2.88±0.11^{b}	2.30±0.26^{a}	7.63±0.29^{ac}
10.0+10.0	66.6±6.6^{b}	2.13±0.25^{a}	4.63±0.52^{a}	2.40±0.23^{b}	1.96±0.42^{a}	6.13±0.20^{ac}
20.0+10.0	60.0±0.0^{b}	1.68±0.12^{a}	2.33±0.37^{a}	1.76±0.08^{b}	1.43±0.20^{a}	5.80±0.17^{ac}
2.5+20.0	46.6±6.6^{b}	1.33±0.29^{a}	2.93±0.46^{a}	1.00±0.11^{a}	1.33±0.08^{a}	3.33±0.20^{a}
5.0+20.0	63.3±3.3^{b}	2.13±0.34^{a}	1.56±0.12^{a}	1.20±0.17^{a}	0.96±0.16^{a}	2.83±0.23^{a}
10.0+20.0	40.0±0.0^{b}	0.80±0.046^{a}	1.23±0.34^{a}	1.00±0.11^{a}	0.63±0.06^{a}	1.66±0.20^{b}
20.0+20.0	-					

'*' indicates mean average values of three repeated experiments with standard error (±SE); (-): No response

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)

Data recorded after 45 days of culture

combination in the MS medium as compared to the control (Table 2.8). A maximum of 6.22 BFC and 13.26 roots were obtained from the explants in MS medium supplemented with a combination of 2.5μM BA and 5μM IAA which was significantly higher than the control. As compared to the control, the length of shoots attained a maximum height of 2.88cm in MS medium supplemented with a combination of 5μM BA and 10μM IAA. However, the length of shoots was suppressed in MS medium supplemented with most of the concentrations of BA and IAA in combinations. Significantly a highest of 4.53cm long roots was obtained in MS medium supplemented with a combination of 2.5μM BA and 5μM IAA as compared to the control. Similarly, a maximum of 8.93% dry matter was formed in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with a combination of 2.5μM BA and 10μM IAA.

Effect of KN + IAA

The multiplication of shoot buds in terms of BFC from the explants of *M. spathulata* was observed to get significantly suppressed in MS medium supplemented with various concentrations of KN and IAA in combination as compared to the control (Table 2.9). However, a maximum of 3.78 BFC was recorded in MS medium supplemented with a combination of 10μM KN and 5μM IAA which was significantly higher than the control. The BFC was inhibited to a minimum of 0.08 in MS medium supplemented with equal concentrations of KN and IAA at 20μM. However, the formation of roots from the *in vitro* shoots was significantly higher with a maximum of 51.23 roots obtained in MS medium supplemented with a combination of 20μM KN and 5μM IAA. Length of the shoots (6.63cm) and roots (2.9cm) were also recorded to be the highest at this particular concentration of KN and IAA in

Table 2.9: Effect of KN and IAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. spathulata*

KN+IAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter * (%)
0.0+0.0	93.3±6.6^{a}	1.42±0.18^{a}	3.00±0.4^{a}	2.6±0.1^{a}	1.6±0.15^{a}	4.2±0.17^{a}
2.5+2.5	53.3±6.6^{b}	1.16±0.14^{a}	6.83±1.15^{a}	1.0±0.09^{b}	0.4±0.01^{b}	3.2±0.05^{a}
5.0+2.5	60.0±0.0^{b}	3.10±0.22^{bc}	7.00±0.95^{a}	1.6±0.11^{a}	1.16±0.03^{a}	4.1±0.16^{a}
10.0+2.5	83.3±6.7^{a}	1.72±0.34^{a}	21.6±0.78^{b}	2.2±0.08^{a}	0.98±0.01^{a}	6.3±0.15^{abc}
20.0+2.5	73.3±6.6^{a}	0.24±0.61^{a}	9.9±0.23^{b}	1.8±0.15^{a}	0.85±0.04^{b}	3.83±0.08^{a}
2.5+5.0	33.3±6.6^{b}	1.01±0.33^{a}	4.8±1.12^{a}	1.6±0.11^{a}	0.52±0.17^{b}	1.46±0.37^{b}
5.0+5.0	56.6±3.3^{b}	3.09±0.45^{bc}	7.43±2.34^{a}	2.0±0.17^{a}	0.59±0.08^{b}	1.73±0.7^{b}
10.0+5.0	66.6±6.6^{b}	3.78±0.71^{bc}	29.5±2.3^{b}	2.96±0.14^{a}	1.37±0.03^{a}	6.9±0.17^{bc}
20.0+5.0	86.6±6.6^{a}	0.88±0.08^{a}	51.23±0.29^{bc}	6.63±0.90^{bc}	2.90±0.11^{bc}	7.2±0.41^{bc}
2.5+10.0	60.0±0.0^{b}	0.92±0.08^{a}	9.73±1.59^{b}	2.95±0.86^{a}	0.88±0.13^{b}	4.0±0.15^{a}
5.0+10.0	63.3±3.3^{b}	1.12±0.22^{a}	10.73±1.73^{b}	5.05±0.37^{b}	0.95±0.02^{b}	5.9±0.06^{abc}
10.0+10.0	60.0±0.0^{b}	0.56±0.20^{a}	14.2±1.06^{b}	3.20±0.11^{a}	1.2±0.05^{a}	4.26±0.43^{a}
20.0+10.0	46.6±6.6^{b}	0.29±0.03^{a}	6.43±0.70^{a}	1.6±0.11^{a}	1.5±0.2^{b}	1.73±0.43^{b}
2.5+20.0	40.0±0.0^{b}	0.66±0.18^{a}	4.10±1.52^{a}	0.2±0.11^{b}	0.3±0.17^{b}	2.06±1.1^{b}
5.0+20.0	53.3±6.6^{b}	0.20±0.14^{a}	6.65±0.31^{a}	1.4±0.11^{a}	0.6±0.11^{b}	1.93±0.57^{b}
10.0+20.0	26.6±6.6^{b}	0.10±0.07^{a}	10.83±0.20^{b}	0.8±0.06^{b}	0.5±0.08^{b}	1.16±0.13^{b}
20.0+20.0	20.0±0.0^{b}	0.08±0.04^{a}	0.00±0.0	0.2±0.09^{b}	0.0±0.0	0.60±0.37^{b}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)
Data recorded after 45 days of culture

Table 2.10: Effect of KN and IAA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. wengeri*

KN+IAA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	2.09±0.44^{a}	4.30±1.05^{a}	2.73±0.29^{a}	1.03±0.14^{a}	4.9±0.14^{a}
2.5+2.5	93.3±6.6^{a}	2.20±0.30^{a}	8.86±0.75^{b}	4.33±0.32^{b}	3.46±0.20^{b}	11.4±0.62^{bc}
5.0+2.5	93.3±6.6^{a}	3.73±0.29^{bc}	12.23±1.15^{bc}	5.73±0.17^{bc}	3.80±0.28^{bc}	12.0±0.45^{bc}
10.0+2.5	93.3±6.6^{a}	4.44±0.36^{bc}	11.26±0.86^{b}	4.46±0.14^{b}	4.86±0.12^{bc}	10.6±0.42^{bc}
20.0+2.5	83.3±6.7^{a}	3.08±0.06^{abc}	10.0±1.11^{b}	4.13±0.29^{b}	4.0±0.23^{bc}	10.3±0.56^{b}
2.5+5.0	86.6±6.6^{a}	3.74±0.53^{bc}	15.8±1.35^{bc}	3.56±0.14^{a}	3.0±0.11^{b}	10.83±0.26^{bc}
5.0+5.0	93.3±6.6^{a}	4.00±0.41^{bc}	9.23±1.02^{b}	4.20±0.15^{b}	3.86±0.23^{bc}	12.13±0.29^{bc}
10.0+5.0	93.3±6.6^{a}	3.36±0.47^{bc}	7.66±0.43^{a}	5.53±0.27^{bc}	4.20±0.40^{bc}	11.06±0.66^{bc}
20.0+5.0	80.0±0.0^{a}	2.46±0.43^{abc}	5.66±0.52^{a}	3.86±0.40^{b}	3.06±0.12^{b}	9.56±0.23^{b}
2.5+10.0	80.0±0.0^{a}	1.38±0.14^{a}	10.8±1.28^{b}	3.76±0.14^{b}	2.30±0.11^{a}	7.73±0.23^{b}
5.0+10.0	73.3±6.6^{a}	2.74±0.18^{abc}	5.53±0.29^{a}	3.00±0.15^{b}	2.66±0.12^{a}	9.23±0.37^{b}
10.0+10.0	66.6±6.6^{b}	1.53±0.27^{a}	3.60±0.61^{a}	2.86±0.21^{a}	1.53±0.13^{a}	8.56±0.08^{b}
20.0+10.0	63.3±3.3^{b}	1.04±0.2^{a}	2.73±0.46^{a}	2.13±0.14^{a}	1.06±0.22^{a}	7.20±0.15^{b}
2.5+20.0	73.3±6.6^{a}	1.68±0.25^{a}	3.13±0.17^{a}	3.10±0.26^{a}	2.40±0.2^{a}	6.23±0.20^{a}
5.0+20.0	73.3±6.6^{a}	1.70±0.41^{a}	3.43±0.24^{a}	3.80±0.20^{a}	1.60±0.21^{a}	3.50±0.17^{a}
10.0+20.0	60.0±6.6^{b}	1.58±0.28^{a}	1.73±0.35^{a}	3.40±0.17^{a}	1.50±0.14^{a}	3.16±0.49^{b}
20.0+20.0	60.0±6.6^{b}	1.56±0.15^{a}	1.20±0.30^{a}	1.70±0.30^{a}	1.20±0.24^{a}	0.97±0.22^{b}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)
Data recorded after 45 days of culture

combination. Except for BFC, the other growth parameters were recorded to be the highest at this concentration of KN and IAA resulting in the accumulation of a maximum of 7.2 % dry matter in the *in vitro* plantlets within 45 days of culture.

The multiplication of shoot buds from the explants of *M. wengeri* in MS medium supplemented with various concentrations of KN and IAA were significantly enhanced as compared to the control. A highest of 4.44 BFC was obtained in MS medium supplemented with a combination of 10μM KN and 2.5μM IAA which was significantly higher than the control (Table 2.10). The BFC got progressively reduced at higher concentration of KN and IAA in the medium. A significant number of 15.8 roots were obtained at 2.5μM KN and 5μM IAA in combination in the MS medium. Shoots attained a maximum length of 5.73cm at 5μM KN and 2.5μM IAA in combination in the medium. Similarly, a maximum of 4.86cm long roots were obtained in MS medium supplemented with a combination of 10μM KN and 2.5μM IAA. A significant amount of dry matter (12.13%) was formed within 45 days of culture in the *in vitro* plantlets with equal concentration of KN and IAA at 5μM in MS medium.

Effect of BA and IBA

The shoot bud multiplication in terms of BFC from the explants of *M. spathulata* was found to get significantly suppressed at various concentrations of BA and IBA in combinations in the MS medium as compared to the control (Table 2.11). However, a maximum of only 2.72 BFC was recorded in MS medium supplemented with 2.5μM BA and 10μM IBA in combination which was significantly higher than the control. A maximum of 10.46 roots were obtained in MS medium supplemented with a combination of 5μM BA and 10μM IBA. The roots were deformed into clump

mass at higher concentrations of BA (2.5μM, 5μM, 10μM) and IBA (20μM) in combination in the medium. Although a maximum of 4.56cm long shoots were obtained in MS medium incorporated with a combination of 10μM BA and 2.5μM IBA, there was a progressive inhibition in the shoot length at higher concentrations of BA and IBA as compared to the control. However, the length of the root were non significant as compared to the control. The *in vitro* plantlets accumulated significantly a maximum of 8.93% dry matter within 45 days of culture in MS medium supplemented with 2.5μM BA and 20μM IBA in combination.

The multiplication of shoot buds from the explants of *M. wengeri* in MS medium supplemented with various concentrations of BA and IBA in combination was recorded to get significantly enhanced (Table 2.12). A maximum of 5.69 BFC was obtained from the explants in MS medium supplemented with a combination of 10μM BA and 2.5μM IBA which was significantly higher than the control. Significant numbers of 11.2 roots were formed from the *in vitro* shoots in MS medium supplemented with a combination of equal concentrations of BA and IBA at 10μM. The shoots attained a highest length of 2.73cm in the control which was significantly higher than the length of shoots obtained in MS medium supplemented with various concentrations of BA and IBA in combinations. However, the roots attained significantly a maximum length of 6.4cm in MS medium supplemented with a combination of 5μM BA and 2.5μM IBA. A maximum of 10.4% dry matter was accumulated in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with a combination of 10μM BA and 2.5μM IBA which was significantly higher than the control.

Table 2.11: Effect of BA and IBA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. spathulata*

BA+IBA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	1.42±0.18^{a}	3.00±0.4^{a}	2.60±0.1^{a}	1.6±0.15	4.2±0.17^{a}
2.5+2.5	73.3±6.6^{a}	1.09±0.30^{a}	3.73±0.06^{a}	1.66±0.20^{b}	1.5±0.15	3.5±0.41^{a}
5.0+2.5	93.3±6.6^{a}	1.80±0.32^{ac}	8.53±0.40^{bc}	2.16±0.20^{b}	1.8±0.28	5.20±0.28^{a}
10.0+2.5	80.0±0.0^{a}	2.02±0.14^{ac}	4.66±0.52^{a}	4.56±0.20^{bc}	1.1±0.15	6.16±0.08^{b}
20.0+2.5	60.0±0.0^{b}	0.76±0.09^{a}	4.00±0.64^{a}	1.63±0.14^{b}	0.6±0.25	3.9±0.11^{a}
2.5+5.0	80.0±0.0^{a}	1.76±0.50^{ac}	3.80±0.23^{a}	1.23±0.95^{b}	2.4±1.05	6.0±0.11^{b}
5.0+5.0	83.3±6.7^{a}	1.98±0.21^{ac}	5.90±0.29^{a}	3.70±0.57 ^{bc}	2.0±0.86	6.23±0.88^{b}
10.0+5.0	80.0±0.0^{a}	1.62±0.04^{ac}	8.40±1.5^{bc}	3.00±0.55 ^{bc}	1.95±0.65	6.06±0.32^{b}
20.0+5.0	66.6±6.6^{b}	0.92±0.09^{a}	4.80±0.69^{a}	2.10±0.60^{b}	1.36±0.26	5.4±0.2^{a}
2.5+10.0	80.0±0.0^{a}	2.72±0.62^{ac}	7.30±0.38^{bc}	2.40±0.51^{bc}	2.0±0.20	4.6±0.3^{a}
5.0+10.0	66.6±6.6^{b}	1.54±0.17^{ac}	10.46±1.15^{bc}	2.70±0.37^{bc}	2.3±0.17	5.7±0.2^{a}
10.0+10.0	53.3±6.6^{b}	1.06±0.23^{a}	5.53±0.17^{a}	3.00±0.08^{bc}	2.1±0.11	6.6±0.18^{b}
20.0+10.0	13.3±6.6^{b}	0.04±0.14^{b}	DCR	0.16±0.24^{a}	-	0.93±0.48^{b}
2.5+20.0	63.3±3.3^{b}	0.68±0.48^{a}	DCR	0.96±0.43^{b}	-	8.93±0.17^{bc}
5.0+20.0	66.6±6.6^{b}	1.30±0.82^{a}	DCR	1.43±0.17^{b}	-	8.66±0.28^{bc}
10.0+20.0	33.3±6.6^{b}	0.20±0.08^{a}	DCR	0.39±0.14 ^{a}	-	7.9±0.1^{bc}
20.0+20.0	-				NS	

'*' indicates mean average values of three repeated experiments with standard error (±SE); DCR: Deformed clump roots; (-): No response; NS: Non-significant

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)

Data recorded after 45 days of culture

Table 2.12: Effect of BA and IBA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. wengeri*

BA+IBA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	2.1±0.44^{a}	4.3±1.05^{a}	2.73±0.29^{a}	1.03±0.14^{a}	4.9±0.14^{a}
2.5+2.5	83.3±6.7^{a}	3.3±0.34^{a}	2.8±0.14^{a}	1.73±0.27^{a}	3.30±0.26^{b}	6.6±0.4^{a}
5.0+2.5	86.6±6.6^{a}	4.05±0.77^{abc}	3.2±0.34^{a}	2.56±0.12^{a}	6.40±0.64^{bc}	9.7±1.04^{bc}
10.0+2.5	90.0±5.7^{a}	5.69±0.51^{bc}	1.6±0.11^{b}	1.20±0.17^{b}	2.20±0.23^{a}	10.4±0.58^{bc}
20.0+2.5	66.6±6.6^{b}	2.6±0.62^{a}	1.0±0.11^{b}	0.63±0.07^{b}	1.93±0.14^{a}	6.03±0.14^{a}
2.5+5.0	80.0±6.6^{a}	2.98±0.23^{a}	3.13±0.29^{a}	1.83±0.26^{a}	2.30±0.15^{a}	5.40±0.26^{a}
5.0+5.0	73.3±6.6^{a}	2.52±0.37^{a}	3.4±0.41^{a}	1.60±0.30^{a}	5.56±0.23^{bc}	9.23±0.26^{bc}
10.0+5.0	73.3±6.6^{a}	1.84±0.28^{a}	1.2±0.11^{b}	0.87±0.08^{b}	1.36±0.08^{a}	8.03±0.43^{bc}
20.0+5.0	66.6±6.6^{b}	1.78±0.30^{a}	0.86±0.17^{b}	0.70±0.09^{b}	0.73±0.10^{a}	6.13±0.48^{a}
2.5+10.0	46.6±6.6^{b}	1.18±0.31^{a}	0.8±0.41^{b}	0.44±0.05^{b}	0.35±0.17^{b}	5.76±0.21^{a}
5.0+10.0	60.0±0.0^{b}	1.88±0.04^{a}	6.2±0.46^{a}	1.06±0.12^{b}	2.83±0.24^{b}	6.80±0.21^{a}
10.0+10.0	66.6±6.6^{b}	2.6±0.38^{a}	11.2±1.09^{bc}	1.90±0.47^{a}	3.73±0.23^{b}	7.73±0.87^{b}
20.0+10.0	80.0±0.0^{a}	5.1±0.14^{bc}	3.93±0.40^{a}	1.46±0.24^{b}	4.46±0.14^{b}	9.70±0.87^{bc}
2.5+20.0	66.6±6.6^{b}	3.82±0.88^{abc}	3.8±0.11^{a}	0.97±0.21^{b}	5.93±0.60^{bc}	10.38±0.73^{bc}
5.0+20.0	73.3±6.6^{a}	2.62±0.3^{a}	3.2±0.30^{a}	1.11±0.30^{b}	2.24±0.17^{a}	9.01±0.53^{bc}
10.0+20.0	73.3±6.6^{a}	1.56±0.37^{a}	1.66±0.35^{b}	2.40±0.15^{a}	2.16±0.43^{a}	5.94±0.07^{a}
20.0+20.0	33.3±6.6^{b}	0.58±0.2^{a}	1.26±0.26^{b}	0.50±0.10^{b}	1.0±0.15^{a}	2.76±0.14^{b}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)
Data recorded after 45 days of culture

Effect of KN and IBA

The multiplication of shoot buds from the explants of *M. spathulata* in MS medium supplemented with various concentrations of KN and IBA in combination varied significantly as compared to the control (Table 2.13). A maximum of only 2.91 BFC was recorded from the explants in MS medium supplemented with a combination 10μM KN and 2.5μM IBA. The multiplication was however significantly inhibited in MS medium supplemented with a combination of KN (2.5, 5, 10, 20μM) and IBA (20μM) at higher concentration. However, a highest of 29.51 roots was formed from the *in vitro* shoots in MS medium supplemented with equal concentrations of KN and IBA at 10μM. The length of the shoots got significantly enhanced to a highest of 6.5cm length in MS medium supplemented with a combination of 5μM KN and 2.5μM IBA. Similarly, the length of the roots were also significantly increased to a highest length of 4.8cm in MS medium supplemented with a combination of equal concentrations of KN and IBA at 5μM. A significant amount of dry matter (7.93%) were formed in the *in vitro* plantlets within 45 days of culture in MS medium supplemented with a combination of 5μM KN and 10μM IBA in the medium.

The multiplication of shoot buds from the explants of *M. wengeri* in MS medium supplemented with various concentrations of KN and IBA in combination was recorded to get significantly increased as compared to the control (Table 2.14). A highest of 4.26 BFC was recorded from the explants in MS medium supplemented with a combination of 20μM KN and 5μM IBA which was significantly higher than the control. A maximum of 13.26 roots were formed from the *in vitro* shoots in MS medium supplemented with a combination of 10μM KN and 2.5μM IBA. The length

Table 2.13: Effect of KN and IBA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. spathulata*

KN+IBA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	1.42±0.18^{a}	3.0±0.4^{a}	2.6±0.1^{a}	1.6±0.15^{a}	4.2±0.17^{a}
2.5+2.5	86.6±6.6^{a}	1.33±0.27^{ab}	16.17±0.62^{b}	4.5±0.23^{b}	3.0±0.05^{b}	6.0±0.05^{abc}
5.0+2.5	90.0±5.7^{a}	2.48±0.11^{ab}	22.71±0.90^{b}	6.5±0.17^{bc}	3.5±0.05^{b}	6.36±0.18^{abc}
10.0+2.5	93.3±6.6^{a}	2.91±0.14^{ab}	26.41±1.06^{bc}	3.33±0.27^{a}	2.7±0.05^{b}	6.2±0.11^{abc}
20.0+2.5	86.6±6.6^{a}	2.27±0.17^{ab}	15.31±1.23^{b}	2.61±0.21^{a}	2.63±0.31^{b}	4.1±0.35^{a}
2.5+5.0	66.6±6.6^{b}	1.43±0.12^{ab}	18.93±1.42^{b}	5.53±0.14^{bc}	3.5±0.05^{b}	5.7±0.86^{abc}
5.0+5.0	86.6±6.6^{a}	2.89±0.22^{ab}	21.7±1.82^{b}	4.96±0.17^{b}	4.8±0.11^{bc}	6.76±0.73^{a}
10.0+5.0	86.6±6.6^{a}	2.36±0.21^{ab}	13.08±0.9^{b}	4.3±0.25^{b}	3.5±0.17^{b}	6.6±1.01^{abc}
20.0+5.0	73.3±6.6^{a}	2.16±1.24^{ab}	10.1±0.51^{b}	4.13±0.12^{b}	2.8±0.15^{b}	4.73±0.51^{a}
2.5+10.0	73.3±6.6^{a}	0.89± 0.21^{a}	18.94±0.87^{b}	5.9±0.20^{bc}	4.53±0.14^{bc}	7.3±0.28^{bc}
5.0+10.0	80.0±0.0^{a}	1.72±0.19^{ab}	25.85±1.63^{bc}	6.3±0.17^{bc}	4.26±0.14^{bc}	7.93±0.57^{bc}
10.0+10.0	66.6±6.6^{b}	1.81±0.17^{ab}	29.51±1.74^{bc}	4.13±0.17^{b}	3.86±0.37^{b}	7.53±1.17^{bc}
20.0+10.0	63.3±3.3^{b}	1.54±0.20^{ab}	10.58±0.87^{b}	4.0±0.11^{b}	2.5±0.17^{a}	7.0±0.3^{abc}
2.5+20.0	60.0±0.0^{b}	0.88±0.02^{a}	9.75±1.42^{b}	3.3±0.40^{a}	2.26±0.24^{a}	4.9±0.34^{a}
5.0+20.0	46.6±6.6^{b}	0.49±0.24^{a}	13.33±1.12^{b}	4.0±0.26^{b}	2.93±0.06^{b}	5.3±0.12^{abc}
10.0+20.0	-					
20.0+20.0	-					

'*' indicates mean average values of three repeated experiments with standard error (±SE); (-): No response

ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)

Data recorded after 45 days of culture

Table 2.14: Effect of KN and IBA incorporated in combination in MS medium on shoot multiplication, growth and development of *M. wengeri*

KN+IBA	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry Matter* (%)
0.0+0.0	93.3±6.6^{a}	2.09±0.44^{a}	4.3±1.05^{a}	2.73±0.29^{a}	1.03±0.14^{a}	4.9±0.14^{a}
2.5+2.5	93.3±6.6^{a}	2.96±0.45^{abc}	6.33±0.24^{a}	3.10±0.11^{a}	5.43±0.14^{bc}	8.1±0.26^{b}
5.0+2.5	93.3±6.6^{a}	3.20±0.4^{abc}	11.13±0.92^{bc}	3.83±0.14^{a}	7.46±0.37^{bc}	12.03±0.67^{bc}
10.0+2.5	86.6±6.6^{a}	3.62±0.39^{abc}	13.26±0.63^{bc}	3.01±0.12^{a}	6.28±0.26^{bc}	10.83±0.86^{bc}
20.0+2.5	66.6±6.6^{b}	2.82±0.19^{abc}	5.76±0.60^{a}	1.07±0.36^{b}	1.42±0.09^{a}	9.60±0.27^{b}
2.5+5.0	73.3±6.6^{a}	1.58±0.25^{a}	9.46±1.36^{abc}	2.43±0.17^{a}	4.66±0.20^{b}	7.73±0.24^{b}
5.0+5.0	86.6±6.6^{a}	2.68±0.36^{abc}	7.33±0.85^{a}	2.65±0.13^{a}	4.85±0.33^{b}	8.93±0.68^{b}
10.0+5.0	93.3±6.6^{a}	3.08±0.37^{abc}	7.0±0.20^{a}	2.87±0.44^{a}	4.41±0.19^{b}	10.0±0.50^{bc}
20.0+5.0	86.6±6.6^{a}	4.26±0.59^{bc}	4.26±0.17^{a}	3.23±0.26^{a}	2.20±0.23^{a}	7.96±0.14^{b}
2.5+10.0	80.0±0.0^{a}	1.32±030^{a}	11.46±0.93^{bc}	1.61±0.11^{a}	2.33±0.39^{a}	8.13±0.20^{b}
5.0+10.0	86.6±6.6^{a}	3.10±0.43^{abc}	12.13±0.70^{bc}	2.40±0.17^{a}	7.0±1.33^{bc}	11.13±0.29^{bc}
10.0+10.0	86.6±6.6^{a}	2.50±0.14^{abc}	10.53±0.65^{bc}	5.30±0.15^{bc}	1.93±0.27^{a}	8.90±0.20^{b}
20.0+10.0	93.3±6.6^{a}	2.01±0.24^{a}	8.06±0.29^{a}	2.10±0.15^{a}	1.66±0.17^{a}	8.50±0.17^{b}
2.5+20.0	93.3±6.6^{a}	2.45±0.36^{abc}	12.06±1.27^{bc}	4.50±0.23^{bc}	6.16±0.20^{bc}	9.30±0.20^{b}
5.0+20.0	93.3±6.6^{a}	2.05±0.55^{a}	8.86±0.88^{a}	4.35±0.13^{bc}	5.60±0.36^{bc}	10.20±0.46^{bc}
10.0+20.0	46.6±6.6^{b}	1.92±0.49^{a}	4.66±0.48^{a}	3.03±0.20^{a}	5.46±0.20^{bc}	8.46±0.14^{b}
20.0+20.0	36.7±3.3^{b}	0.29±0.09^{a}	3.0±0.46^{a}	2.56±0.39^{a}	2.30±0.12^{a}	6.70±0.20^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of control and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)
Data recorded after 45 days of culture

of the shoots got significantly increased to a maximum of 5.3cm in MS medium supplemented with equal concentrations of KN and IBA at10μM. Similarly, the roots attained a highest of 7.46cm length in MS medium supplemented with a combination of 5μM KN and 2.5μM IBA. The dry matter content of the *in vitro* plantlets got increased to a maximum of 12.03% within 45 days of culture in MS medium supplemented with a combination 5μM KN and 2.5μM IBA.

B) *Effect of photoperiod*

The multiplication of shoot buds in terms of BFC from the explants of *M. spathulata* varied significantly under different photoperiods with a highest of 5.8 BFC at 12h photoperiod within 45 days of culture in the optimum multiplication medium (MS medium supplemented with a combination of 10μM BA and 2.5μM NAA). Maximum of and 19.3 roots were obtained at 12h photoperiod which was the highest as compared to rest of the photoperiods (Table 2.15; Fig. 4 B). The multiplication of shoot buds from the explants was suppressed to a minimum of 1.3 BFC at 24h photoperiod. However, the length of the shoots (6.3cm) and roots (6cm) recorded at 18h photoperiod were significantly higher as compared to the rest of the photoperiods. The *in vitro* plantlets accumulated a maximum of 7.8% dry matter within 45 days of culture at 12h photoperiod which was the highest as compared to other photoperiods.

The multiplication of shoot buds from the explants of *M. wengeri* under different photoperiods varied significantly under different photoperiods with a maximum of 7.2 BFC at 12h photoperiod within 45 days of culture in the optimum multiplication medium (MS medium supplemented with a combination of 5μM BA and 2.5μM NAA). Around 21.9 roots were recorded at 12h photoperiod which was the highest as compared to the rest of the photoperiods tried (Table 2.16; Fig. 4 D).

Table 2.15: Effect of photoperiod on shoot multiplication, growth and development of *M. spathulata* in MS medium supplemented with BA (10μM) and NAA (2.5μM) in combination

Photoperiod (h) (24°C, 40.5μmoles $m^{-2}s^{-1}$)	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry matter* (%)
0.0	33.3±6.6^{a}	0.63±0.44^{a}	2.3±0.8^{a}	0.7±0.17^{a}	0.56±0.56^{a}	2.0±0.45^{a}
6.0	60.0±11.5^{abc}	1.5±0.79^{a}	7.6±1.1^{b}	1.2±0.34^{b}	0.83±0.35^{a}	3.3±0.34^{a}
12.0	93.3±6.6^{bc}	5.8±0.72^{bc}	19.3±0.7^{bc}	3.8±0.63^{bc}	2.56±0.75^{a}	7.8±1.0^{bc}
18.0	86.6±6.6^{bc}	3.9±0.65^{bc}	12.2±1.0^{bc}	6.3±0.76^{bc}	6.0±0.64^{bc}	6.4±0.37^{bc}
24.0	66.6±6.6^{abc}	1.3±0.15^{a}	5.2±1.8^{a}	2.0±0.20^{a}	4.1±1.1^{bc}	2.6±0.4^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of photoperiod (dark/0h) and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 45 days of culture

Table 2.16: Effect of photoperiod on shoot multiplication, growth and development of *M. wengeri* in MS medium supplemented with BA (5μM) and NAA (2.5μM) in combination

Photoperiod(h) (24°C, 40.5μmoles $m^{-2}s^{-1}$)	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry matter* (%)
0.0	36.6±3.3^{a}	0.29±0.05^{a}	4.66±1.15^{a}	0.96±0.29^{a}	0.33±0.14^{a}	2.86±0.40^{a}
6.0	93.3±6.6^{bc}	3.94±0.8^{b}	9.63±1.65^{a}	3.0±0.56^{b}	1.56±0.31^{a}	5.1±0.43^{b}
12.0	93.3±6.6^{bc}	7.2±0.86^{bc}	21.9±4.3^{bc}	3.26±0.48^{b}	2.93±0.1^{b}	7.93±0.4^{bc}
18.0	53.3±6.6^{a}	1.0±0.09^{a}	23.1±4.6^{bc}	6.36±0.44^{bc}	5.23±0.5^{bc}	4.16±0.61^{a}
24.0	46.6±6.6^{a}	0.90±0.12^{a}	6.93±0.70^{a}	1.23±0.20^{a}	0.8±0.17^{a}	3.66±0.35^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of photoperiod (dark/0h) and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 45 days of culture

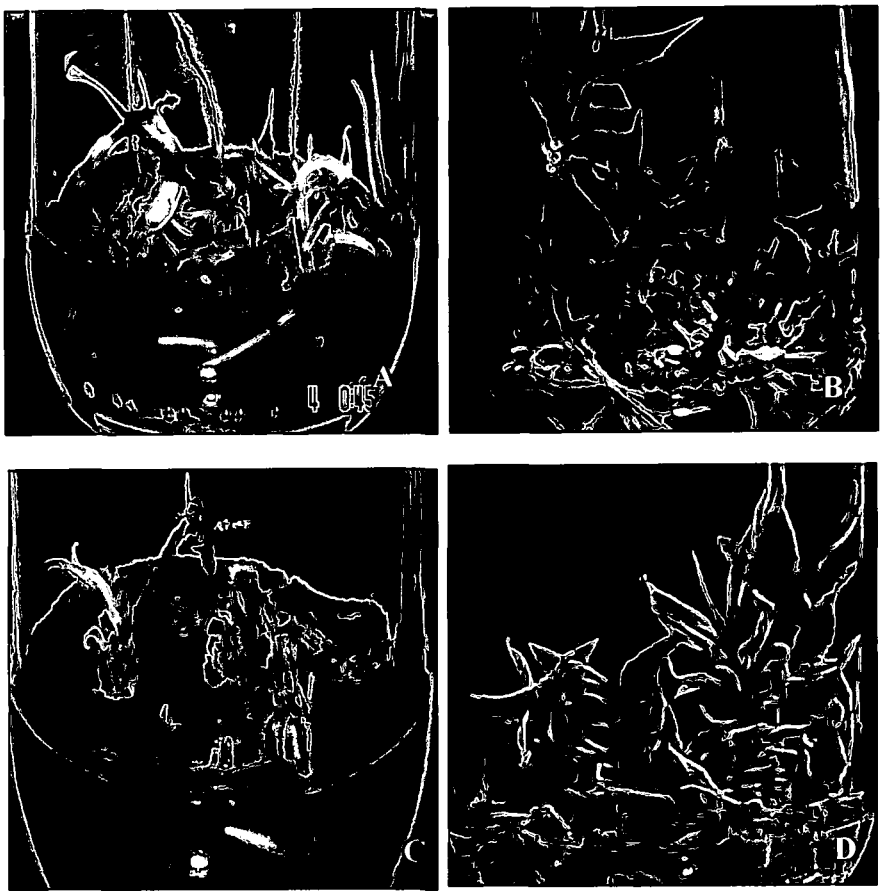


Fig. 4

The multiplication of shoots from the explants was suppressed to a minimum of 0.9 BFC at 24h photoperiod. However, the shoots as well as roots attained a maximum of 6.36cm and 5.23cm length respectively at 18h photoperiod within 45 days of culture. Dry matter of around 7.93% was accumulated in the *in vitro* plantlets within 45 days of culture at 12h photoperiod which was significantly higher.

C) *Effect of temperature*

The explants of *M. spathulata* failed to multiply in the optimum multiplication medium (MS medium supplemented with a combination of 10μM BA and 2.5μM NAA) incubated at a temperature of 0°C. The multiplication of shoots from the explants gradually increased at higher temperatures. Multiplication of shoots was observed to be significantly higher at 24°C in the optimum medium with a maximum of 6.28 BFC (Table 2.17; Fig. 5 B). However, the multiplication was inhibited to a minimum of only 0.1 BFC at 30°C. The number of roots (19.4), the shoot length (3.76cm), root length (2.93cm) and dry matter content (7.8%) of the *in vitro* plantlets were recorded to be the highest at 24°C as compared to other ranges of temperatures. However at 30°C, these parameters of growth of *in vitro* plantlets were observed to be significantly inhibited to their minimum.

The multiplication of shoot buds from the explants of *M. wengeri* were completely inhibited at 0°C in the optimum multiplication medium (MS medium supplemented with a combination of 5μM BA and 2.5μM NAA). However, multiplication of shoot buds from the explants gradually increased at higher temperatures. Multiplication of shoot buds was observed to be the highest at 24°C with a maximum of 7.96 BFC in the optimum medium (Table 2.18; Fig. 5 D). However at 30°C, the multiplication of shoots was inhibited to minimum of 0.62 BFC

Table 2.17: Effect of temperature on shoot multiplication, growth and development of *M. spathulata* in MS medium supplemented with BA (10μM) and NAA (2.5μM) in combination

Temperature (°C) (12h, 40.5μmoles $m^{-2}s^{-1}$)	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry matter* (%)
0 °C	-	-	-	-	-	-
4°C	26.7±6.6^{a}	0.026±0.0^{a}	0.26±0.0a	0.066±0.0 ^{a}	0.16±0.0^{a}	0.53 ± 0.0^{a}
8°C	33.3±6.6^{a}	0.34±0.14^{a}	4.26±2.2a	0.59±0.17^{a}	0.56±0.29^{a}	1.96±0.49^{a}
16°C	73.3±6.6^{bc}	1.94±0.46^{a}	7.6±1.83^{b}	1.68±0.56^{a}	1.72±0.2^{ac}	5.5±0.61^{bc}
24°C	93.3±6.6^{bc}	6.28±1.0^{b}	19.4±2.8^{bc}	3.76±0.52^{b}	2.93±0.3^{bc}	7.8±1.37^{bc}
30°C	26.6±6.6^{a}	0.10±0.06^{a}	0.0±0.0	0.16±0.05	0.0±0.0	0.53±0.14^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of 4°C and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 45 days of culture

Table 2.18: Effect of temperature on shoot multiplication, growth and development of *M. wengeri* in MS medium supplemented with BA (5μM) and NAA (2.5μM) in combination

Temperature (°C) (12h, 40.5μmoles $m^{-2}s^{-1}$	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry matter* (%)
0 °C	-	-	-	-	-	-
4°C	13.3±6.6^{a}	0.06±0.03^{a}	0.2±0.02^{a}	0.18±0.09^{a}	0.07±0.01^{a}	0.72±0.37^{a}
8°C	46.6±6.6^{b}	0.48±0.12^{a}	0.8±0.46^{a}	0.5±0.10 ^{a}	0.41±0.21^{a}	5.4±0.58^{bc}
16°C	80.0±0.0^{b}	2.29±0.32^{a}	10.1±1.4^{b}	1.28±0.24^{b}	1.75±0.5^{b}	8.16±1.0^{bc}
24°C	93.3±6.6^{bc}	7.96±1.6^{b}	20.7±2.8^{bc}	3.03±0.37^{bc}	3.2±0.37^{bc}	8.56±0.8^{bc}
30°C	46.6±6.6^{bc}	0.62±0.23^{a}	8.73±0.7^{b}	0.76±0.14^{a}	0.83±0.17^{a}	1.8±0.50 ^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of 4°C and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 45 days of culture

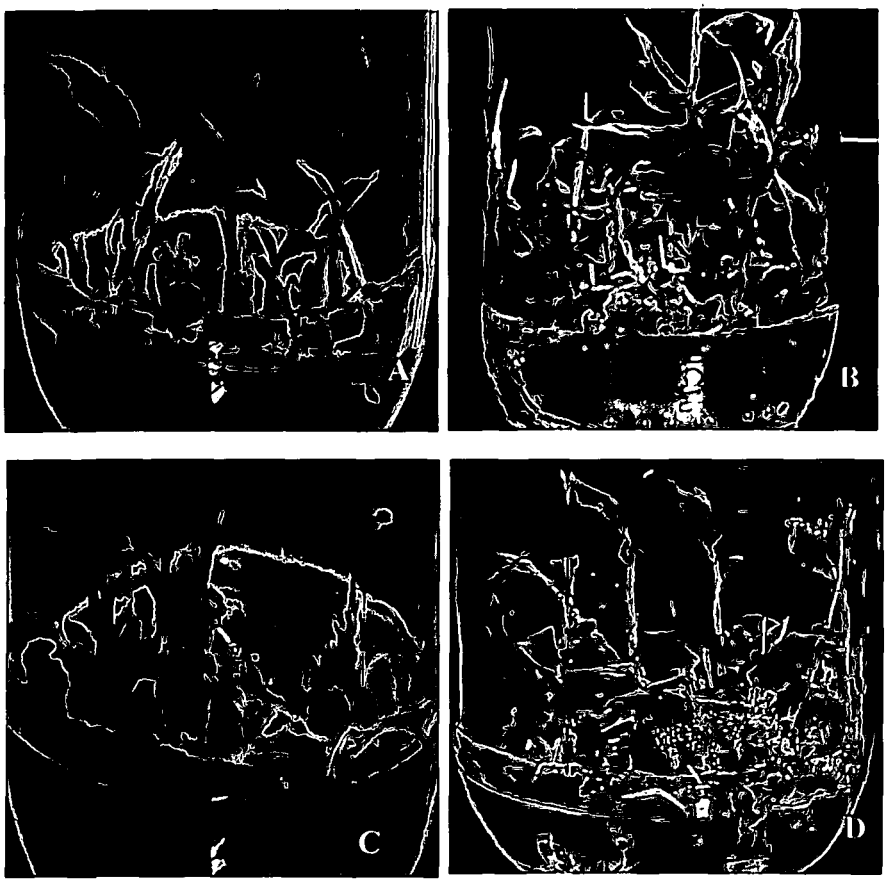


Fig. 5

in the optimum medium. The number of roots (20.7), the shoot length (3.03cm), root length (3.2cm) and dry matter content (8.56%) of the *in vitro* plantlets were significantly highest at 24°C in the optimum multiplication medium as compared to other ranges of temperature. At 30°C, these parameters of growth of the *in vitro* plantlets were recorded to get significantly inhibited.

D) *Effect of light*

The multiplication of shoot buds from the explants of *M. spathulata* in the optimum multiplication medium subjected to a very low light intensity (0.0 - 0.027μmoles m^{-2} s^{-1}) was significantly very less with only 0.35 BFC within 45 days of culture. The shoot bud multiplication from the explants was gradually enhanced at higher intensities of light. The multiplication of shoot buds got significantly enhanced to a maximum of 5.72 BFC at 40.5μmoles m^{-2} s^{-1} in the optimum multiplication medium within 45 days of culture (Table 2.19; Fig. 6 B). The root number (18.86) and shoot length (3.68cm) of the *in vitro* plantlets were also recorded to be significantly highest at 40.5μmole m^{-2} s^{-1} light intensity as compared to other light intensities. However, the length of the roots (3.55cm) and the dry matter content (11.5%) were significantly higher at 27μmoles m^{-2} s^{-1} light intensity. The BFC, root number, shoot and root length and the dry matter content of the *in vitro* plantlets were significantly suppressed to minimum at 54μmoles m^{-2} s^{-1} light intensity in the optimum multiplication medium.

The multiplication of shoot buds from the explants of *M. wengeri* in the optimum multiplication medium subjected to a very low light intensity (0.0 - 0.027μmole m^{-2} s^{-1}) recorded a minimum of only 0.22 BFC within 45 days of culture. The shoot multiplication from the explants was gradually enhanced at higher

Table 2.19: Effect of light on shoot multiplication, growth and development of *M. spathulata* in MS medium supplemented with BA (10μM) and NAA (2.5μM) in combination

Light μmoles $m^{-2}s^{-1}$ (24°C, 12h)	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry matter* (%)
0.0 - 0.027	33.3±6.6^{a}	0.35±0.07^{a}	3.6±0.75^{a}	0.37±0.13^{a}	0.46±0.24^{a}	2.1±0.47^{a}
13.5	53.3±6.6^{a}	0.86±0.38^{a}	5.2±1.47^{a}	0.68±0.18^{a}	0.90±0.15^{a}	8.6±0.58^{bc}
27.0	86.6±6.6^{bc}	4.25±0.4^{bc}	10.4±1.29^{bc}	2.74±0.47^{abc}	3.55±0.4^{bc}	11.5±1.4^{bc}
40.5	93.3±6.6^{bc}	5.72±0.6^{bc}	18.86±1.4^{bc}	3.68±0.81^{bc}	3.2±0.63^{bc}	7.6±1.2^{bc}
54.0	73.0±6.6^{bc}	2.3±0.70^{a}	6.5±0.70^{a}	2.5±0.52^{abc}	2.6±0.8^{abc}	6.0±0.56^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of dark (0.0 - 0.027) and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p=0.05)
Data recorded after 45 days of culture

Table 2.20: Effect of light on shoot multiplication, growth and development of *M. wengeri* in MS medium supplemented with BA (5µM) and NAA (2.5µM) in combination

Light µmoles $m^{-2}s^{-1}$ (24°C, 12h)	Response* (%)	BFC*	Root no.*	Shoot length (cm)*	Root length (cm)*	Dry matter* (%)
0.0 - 0.027	20.0±11.5^{a}	0.22±0.13^{a}	2.13±1.21^{a}	0.56±0.34^{a}	0.4±0.23^{a}	3.06±0.29^{a}
13.5	80.0±0.0^{bc}	3.28±0.16^{abc}	5.86±1.0^{a}	1.54±0.13^{a}	3.23±0.7^{bc}	11.5±1.0^{bc}
27.0	80.0±0.0^{bc}	3.41±0.86^{abc}	14.76±2.0^{bc}	2.23±0.31^{bc}	4.46±0.7^{bc}	13.7±1.6^{bc}
40.5	93.3±6.6^{bc}	7.26±1.24^{bc}	20.26±1.6^{bc}	3.1±0.27^{bc}	3.0±0.4^{abc}	8.4±0.4^{abc}
54.0	66.6±6.6^{bc}	2.14±0.12^{a}	4.93±0.86 ^{a}	1.8±0.32^{a}	2.9±0.7^{abc}	7.7±0.8^{abc}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means of dark (0.0 - 0.027) and the highest value compared with rest of the values and followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p=0.05)
Data recorded after 45 days of culture

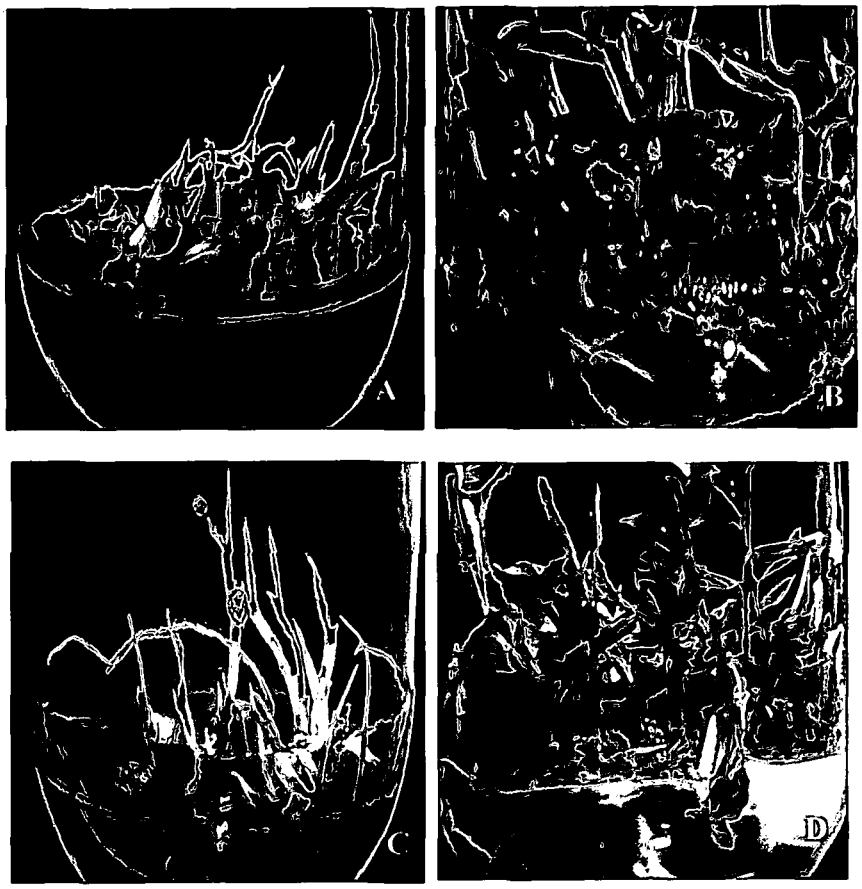


Fig. 6

intensities of light. The multiplication of shoots got significantly enhanced to a maximum of 7.26 BFC at 40.5μmoles m^{-2} s^{-1} in the optimum multiplication medium within 45 days of culture (Table 2.20; Fig. 6 D). The number of root (20.26) and length of shoot (3.1cm) of the *in vitro* plantlets were recorded to be the highest at 40.5μmoles m^{-2} s^{-1} light intensity) in the optimum multiplication medium. However, the length of the roots (4.46cm) and the dry matter content (13.7%) were the highest at 27μmoles m^{-2} s^{-1} light intensity. With further increase in light intensity to a maximum of 54μmoles m^{-2} s^{-1}, the BFC, root number, shoot and root length and the dry matter content of the *in vitro* plantlets got suppressed to minimum.

DISCUSSION

The beautiful plants of both the species of *Mantisia* were identified in the blooming period and were collected from their wild habitats. There has been no report on collection of *M. spathulata* and *M. wengeri* from their wild habitats in the past several years. It was noticed that the natural habitats have undergone severe landslides at different places and therefore, the two species are facing a high risk of becoming extinct in near future with the occurrence of any major landslide. Therefore, from the present study it was found that one crucial factor for the depletion of the plants is the occurrence of frequent landslides in the natural habitats. The collection of these two species from their natural habitats became very significant in this study from the conservation point of view.

The selection of explants is the most important factor that should give maximum regeneration in terms of shoots or morphogenetic response within a short time. In the present study, the juvenile leaves and roots used as explants failed to give

any response. Only rhizome buds were found to be most suitable explants for *in vitro* regeneration of plantlets. The use of rhizomatous buds as explants has been reported in many zingibers as well as hydrophytes. The maximum response from these explants might be due to the presence of apical and cambial meristems inside these tissues, as opined by various workers (Murashige, 1974; Ammirato, 1983; Bajaj 1986b; Gamborg and Phillips, 1986). Several trials were carried out for surface sterilization of the explants and only 20 - 40% of the *in vivo* rhizomatous explants for both the species could be made free of contamination by surface sterilizing with 0.1% $HgCl_2$ for 5min. The inherent difficulties in the establishment of axenic cultures have been attributed to the presence of large bacterial and fungal populations, endophytic organisms in highly lacunate tissues and lack of cutinized epidermis (Madsen, 1985; Godmaire and Nalewajko, 1986). The selection of sterilizing agent depends upon various factors like nature of explants (soft/hard, underground/overground), extent of surface microflora, and the sensitivity of the explant tissues to various sterilizing agents. The sterilization of explants is possible by repeated hit and trial methods. Duration of surface sterilization with sterilant varies with different species or nature of explants used for culture (Sakamura and Suga, 1989). In the present study, the establishment of aseptic cultures was difficult, but once a healthy culture was established, there was no further contamination. Similar findings have been observed with ginger by earlier investigators (Hosoki and Sagawa, 1977; Inden and Asahira, 1988; Balachandran *et al.*, 1990).

Out of the various media investigated, the initiation of shoot buds from the cultured explants was recorded only in MS medium supplemented with growth regulators and therefore, MS medium was used for carrying out subsequent

experiments. Major reduction in the level of nutrients mainly nitrogen in B_5, M and KC media might have adversely affected the shoot bud induction. MS medium has been found to be the most suitable medium for the micropropagation for a large number of Zingiberaceae plants (Nadgauda *et al.*, 1978; Yasuda *et al.*, 1988; Reghunath and Gopalakrishnan 1991; Sharma and Singh, 1995; Anand and Hariharan 1997; Shirin *et al.*, 2000; Prathanturarug *et al.*, 2004; Stanly and Keng, 2007; Miceli *et al.*, 2008). As nitrogen is well known to serve as a constituent of many plant cell components, its deficiency inhibits plant growth. In addition to the total nitrogen content, the ratio of nitrate to ammonium (NH_4^+) is a very important aspect in nitrogen nutrition (Ramage *et al.*, 2002). This is because of the fact that the ratio strongly influences pH of the medium, which in turn determines the absorption of different nutrients. Thus, as in most plant species (Tefera and Wannakrairoj, 2004), the relatively higher supply of nitrate-nitrogen within the MS medium could have exerted the profound effect on shoot initiation and growth in the plants studied.

The plant growth regulators are the major catalyst for alternating the response of tissues to any type of synthetic medium and play a key role in determining the *in vitro* morphogenesis. The incorporation of growth regulators viz. cytokinins (BA, KN) and auxins (IAA, IBA, NAA) in the MS medium at various concentrations resulted in differential response with regard to multiple shoot bud induction, growth and development of plantlets. In the present study, using 5μM and 10μM of BA in combination with a reduced concentration of NAA (2.5μM) in the MS medium had promotory effect on shoot bud proliferation for both *M. spathulata* and *M. wengeri* respectively. In many other species of zingibers, such as in *Kaempferia galanga*, the addition of 12μM BA in combination with 3μM NAA to the medium significantly

enhanced the shoot bud proliferation (Shirin *et al.*, 2000). Highest shoot bud induction rate of 6.1 shoots per explant in *Zingiber petiolatum* was recorded in MS medium with NAA at 0.5µM in combination with 17.8µM BA (Prathanturarug *et al.*, 2004). Similarly, *Curcuma amada* rhizome buds produced 7 - 12 shoots and roots simultaneously in MS medium fortified with $0.5mgl^{-1}$ NAA and $4mgl^{-1}$ BA in combination (Borthakur and Bordoloi, 1992). In the present study, it was observed that the use of low concentration of NAA increased the multiplication of shoots. This in agreement with the findings of Han *et al.* (1994), who reported that using BA with NAA at low concentration have significant promontory effect on rhizome bud formation as compared to using BA singly. The superior effects of BA have been emphasized earlier in many medicinal zingibers as well such as *Zingiber officinale* and *Curcuma* species (Balachandran *et al.*, 1990; Miachir *et al.*, 2004). Cytokinins as a plant growth regulator causes shoot induction by stimulating cell division and decreasing apical dominance (Shekafandeh and Khosh-Khui, 2007). KN in the medium has been reported to have the ability to induce multiple shoots from the explants of many zingibers such as *Alpinia galanga* (Borthakur *et al.*, 1999). Nadgauda *et al.* (1980) obtained healthy shoots of ginger, when cultivated in MS medium supplemented with KN. Similarly, the synergistic effect of KN and NAA has been demonstrated in *Zingiber officinale* (Sharma and Singh, 1997). The application of KN in combination with NAA showed moderate response and shoot multiplication from the explants of *M. spathulata* and *M. wengeri*. However, the growth and development of plantlets in KN supplemented medium in combination with all other auxins augmented the shoot length and dry matter accumulation in the regenerants of both the species. This is in accordance with the report on *Hedychium spicatum* where

shoot bud proliferation was inhibited using but formation of roots was enhanced in KN containing medium (Koul *et al.*, 2005). Most of the micropropagation reports (68%) shows the superior effect of BA to the promotive effect of KN on micropropagation (Hu and Wang, 1983).

Auxins are a group of plant growth regulators which generally produce adventitious roots from the shoots. However, auxins at higher concentration may also induce callus from the explants in many cases. Unlike the occurrence of absolute concentration of the plant growth regulators, it is the balance between auxin and cytokinin that regulates the growth and differentiation (Evans *et al.*, 1983; George, 1993). In the present study, coupling of auxin (NAA) with cytokinin (BA) induced more number of shoots as compared to the use of cytokinin alone in the medium. The multiplication of shoot buds for both *M. spathulata* and *M. wengeri* got reduced in MS medium supplemented singly with auxins (NAA, IBA and IBA). However, auxins (NAA, IBA, and IAA) used singly in the medium stimulated sufficient number of roots with maximum number being obtained in NAA supplemented medium. At very high concentrations of auxins (NAA and IBA) alone and in combination with cytokinins (BA or KN) in the medium, deformation of the roots forming callus at the later stage (after 120 days of culture) was observed. Hu and Wang (1983) suggested that, though exogenous auxins did not generally promote axillary shoot proliferation, culture growth could be improved by its presence. One of the possible roles of auxins in the shoot multiplication stage is to nullify the suppressive effect of high cytokinin in the medium, when these are present together. Many reports are available, in which auxin-cytokinin combinations favour shoot multiplication. For instance, Priyadarshan *et al.* (1988) succeeded in the clonal propagation of different cultivars of Cardamom

in MS medium, fortified with IAA and BA. Induction of shoots and roots were significantly enhanced in *Alpinia subulatum*, when BA and IAA were added in combination to the MS medium (Sajina *et al.*, 1997). Multiple shoot cultures of *Hedychium spicatum* were established in MS medium supplemented with BA and IAA from the pre-existing buds on the rhizome (Koul *et al.*, 2005). Synergistic effect of IBA at 2.5μM and BA (4.4μM) for *in vitro* regeneration, growth and development of *Curcuma Zedoaria* (Stanly and Keng, 2007) and also in *Renealmia mexicana* (Miceli *et al.*, 2008) has been reported in recent years. In the present study, the roots of the *in vitro* plantlets of *M. spathulata* and *M. wengeri* attained maximum length in medium supplemented with IBA in conjugation with either BA or KN in comparison to NAA and IAA. Similarly, Bunn and Dixon (1992) reported that IBA was effective in stimulating root development and elongation for *Grevillea scapigera,* an endangered Australian species.

In plant tissue cultures, an external factor such as light (wavelength, light intensity and photoperiod) plays an important role, in addition to media composition (Hughes, 1981; Hussey, 1986). These light factors are not independent but interact in complex ways (Hughes, 1981). Responses of plant tissue cultures during organogenesis to photoperiod and light intensity may be species-, variety- and explant-dependant (Economou and Read, 1987). In the present investigation, a very low light intensity or at complete darkness i.e. (0.0 - 0.027μmoles $m^{-2}s^{-1}$), initiation of shoot buds and the growth and development of plantlets was very low for *M. spathulata* and *M. wengeri*. The shoots were etiolated and remained curled with pale unopened leaves. With increase in the light intensity the shoot proliferation, growth and development progressively increased in both the species. However, beyond an

optimum level, there was a progressive decrease in the shoot proliferation, growth and development. Explants cultured at 40.5μmoles $m^{-2}s^{-1}$ light intensity were found to result in highest regeneration of shoot buds in both the species. However, root length and dry matter accumulation was more at 27μmoles $m^{-2}s^{-1}$ light intensity.

Generally, plant growth and development are affected by both internal factors including genotype and plant hormones, and external factors such as, light, temperature, photoperiod etc. Light is the ultimate substrate for photosynthetic energy conversion, and can also harm the plants. Higher light intensity causes photo oxidation which involves the destruction of chlorophyll, resulting in less biomass production. High light intensity is damaging to the water-splitting photosystem II (PSII), leading to the degradation of the reaction center. The frequency of this damage is relatively high when light intensity is increased, especially when combined with other environmental factors (Soontornchainaksaeng *et al.*, 2001). There is an exception for lily plants in which no photo inhibition or damage to PSII was observed in the critical condition of strong light and high temperature in the culturing season (Sorentino *et al*, 1997). Plant growth is related to the function of growth hormones like auxin, which is sensitive to high light intensity. Cytokinins act in concert with auxin to cause cell division in plant tissue culture. In moderate light intensity, plants generally bear longer internodes, and are less tough and more succulent with larger leaves than those grown in intense light (Barber and Anderson, 1992). In papaya, the root formation of shoots or embryoids derived from callus or shoot tips occurred at light intensities of 3000 - 4000 lux (Yie and Liaw, 1997). The best plant height and leaf length in *Vanda coerulea* were found in plantlets exposed to 37μmoles $m^{-2}s^{-1}$ light intensity (Soontornchainaksaeng *et al.*, 2001). Similarly, a photosynthetic photon

flux density (PPFD) of 33.75 - 45µmoles $m^{-2}s^{-1}$ and 45µmoles $m^{-2}s^{-1}$ intensity was found optimum for shoot proliferation, growth and development of *Chlorophytum arundinaceum* and *Malus zumi* plantlets respectively (Lattoo *et al.*, 2005; Xu *et al.*, 2008).

The photoperiod seems to influence the rate of multiplication of shoot buds and growth of the plantlets in *M. spathulata* and *M. wengeri*. The *in vitro* photoperiod requirements vary from one plant species to other. Shoot bud regeneration was reported to be highest under 24h illuminations in the species of Brassica (Jain *et al.*, 1988) and ginger (Rout and Das, 1998) whereas in lettuce cultures 16h photoperiod was found to be optimum (Kadkade and Seibert, 1977). In *Chenopodium rubrum* growth was stimulated with increase in day length and the plants attained maximum height under 24h long day photoperiod for 10 weeks (Mitrovic and Culafic, 2007). In the present study, 12h photoperiod with 40.5µmoles $m^{-2}s^{-1}$ was found to be suitable to influence shoot number and dry matter accumulation as compared to other photoperiodic durations. However, at 18h photoperiod the shoot and root length of the regenerants were maximum for both the species. In many species, the length of the photoperiod is an important primary signal for the growth responses (Li *et al.*, 2003). Long day photoperiod (LD) sustains shoot elongation, whereas short photoperiod (SD) induces growth cessation and formation of terminal buds (Junttila, 1976; Howe *et al.*, 1995). Maximum regeneration and better growth of several plants such as *Curcuma longa* (Nadgauda *et al.*, 1978; Salvi *et al.*, 2001) and *Calophyllum apetalum* (Nair and Seeni, 2003) has been reported at equal durations of light and day. In the present study, it was found that at 24h photoperiod, the proliferation of shoots and the growth of the *in vitro* regenerants got significantly inhibited.

The influence of temperature on basic physiological processes such as respiration and on cell and organ formation is well known (Reed, 1992). In the present study, it was found that at very low temperature (4, 8°C) minimal response was obtained from the cultured explants. However, increasing the temperature to 24°C the response from the explants was enhanced and thereby the overall growth and regeneration increased for both the regenerants of *M. spathulata* and *M. wengeri*. Temperature of around 25±2°C has been found to be optimum for better growth and regeneration in most of the plants including the Zingiberaceae species (Miachir *et al.*, 2004; Koul *et al.*, 2005; Stanly and Keng, 2007; Miceli *et al.*, 2008). Low temperatures may affect the growth and reproduction capacities of plants by limiting photosynthesis through what is known as the chilling-dependent photoinhibition of photosynthesis (Powles, 1984; Oquist *et al.*, 1987; Baker *et al.*, 1988) mainly inhibiting the photosystem II complex and specific degradation of D1 protein (Osmond, 1981; Long, 1983; Powles, 1984; Asada, 1994; Miyao, 1994). At 30°C, there was a progressive decrease in the response from the explants leading to minimum shoot proliferation and growth of the *in vitro* regenerants. However, *in vitro* regeneration and organogenesis in *Archis hypogaea* was found to be better at 28°C and 35°C (Pestana *et al.*, 1999). Thus, from the present study it becomes clear that it is crucial to check the optimal physico-chemical requirements for the growth of the regenerants which might vary for different plant species and the type of germplasm used.

CHAPTER III: HARDENING AND ACCLIMATIZATION OF *IN VITRO* RAISED PLANTLETS OF *MANTISIA SPATHULATA* AND *MANTISIA WENGERI*

INTRODUCTION

Plantlets often die during the transfer from *in vitro* to *ex vitro* conditions (Pospisilova *et al.*, 1999). The overall success of *in vitro* raised plantlets depends on successful hardening and transplantation in the field. Under controlled culture conditions the anatomical and morphological conditions of *in vitro* plantlets such as development of cuticle, hairs, opened stomata photosynthetic ability and conducting tissues etc. required for the growth and development of plantlets remains non functional. The *in vitro* plantlets are very delicate and therefore wilt rapidly on direct transfer to normal green house or field condition. Rapid loss of water through transpiration (Grout and Aston, 1977) may lead to high mortality rate unless plantlets are transferred gradually from initial high humidity to reduced humidity and increased light intensity (George and Sherrington, 1984). However, acclimatization of *in vitro* raised plantlets prior to transfer helps the plants to adapt to the environmental changes (Brainerd and Fuchiagam, 1981; Roy, 1994; Baruah, 1996). Bhojwani and Razdan (1983) have stressed on high humidity conditions during the initial days for successful transplantation. Therefore, for successful transplantation the first and foremost requirement is the maintenance of plantlets under very high humidity conditions (90 -

100%) for the initial 10 - 15 days followed by gradual reduction of humidity (70 - 60%) and temperature (28 - 38°C) in the glasshouse (Vij *et al.*, 1995). Temperature is also very crucial for higher survival rate and growth of transplanted plants. During summer, plants are exposed to high irradiance and temperature (30 - 40°C) and low humidity. Thus, careful step wise procedure is needed when *in vitro* plants are transferred to pots or field conditions.

For hardening of *in vitro* plantlets, various compositions of substrates such as mixtures of sand, charcoal, brick pieces, dry cow dung etc. in different ratios have been tried out for good drainage and sufficient aeration of roots in wide range of plants including many orchids (Kumaria and Tandon, 1994; Kumaria *et al.* 2005). However, the available literature shows that most of the species of zingibers are easily hardened in soil and sand mixtures (Nadgauda *et al.*, 1978; Borthakur *et al.*, 1999; Prathanturarug *et al.*, 2004) and some times farmyard manure proves to be beneficial for higher survivability and better growth of the transferred plantlets (Shirin *et al.*, 2000). Proper drainage prevents plantlets from the fungal infection and, aeration provides formation of cuticle and waxes over the roots thereby increasing the capacity of roots for nutrient uptake from the potting substrates. Different types of pots have been used for acclimatization of plantlets, but the glazed pots are not suitable, as they do not allow sufficient aeration of the roots and the compost. Mukherjee (1983) suggested the use of clay pots for many epiphytic orchids like *Cattleya, Epidendrum, Dendrobium* etc. To facilitate drainage and aeration, the plastic pots are poked for small holes. High humidity is generally maintained by covering the transferred plantlets with perforated polybags which are removed after few days resulting in

decrease in humidity leading to the gradual acclimatization of plantlets within 3 - 4 weeks of transfer (Bisht *et al.*, 1988; Palini *et al.*, 1994; Vyas *et al.*, 1999).

Preconditioning of *in vitro* cultured plantlets has been useful for successful acclimatization of plantlets in the field. Hazarika *et al.* (2000, 2001) reported that *in vitro* preconditioning of citrus microshoots with sucrose concentrations of 3% was optimum for subsequent *ex vitro* survival and growth. Similarly, Nagaraju and Mani (2005) reported an *in vitro* prehardening of *Zygopetelum intermedium* in medium containing paclobutrazol and activated charcoal for its high rate *ex vitro* survival and growth of plantlets.

MATERIALS AND METHODS

Healthy *in vitro*-raised complete plantlets of both *M. spathulata* and *M. wengeri* were taken for hardening and establishment. Eight weeks- old plantlets measuring 2.5 - 3cm in height were taken out from the culture tubes/flasks by means of long handled spoon along with a small amount of the adhering agar. The agar medium sticking to the roots was removed slowly with a soft brush. The plantlets were washed with sterile water taking due care to avoid damage to the roots. These were then transferred to clean 8cm long thermocol/plastic pots containing different mixtures of composts viz., (i) soil, sand and compost (dry cow dung) (1: 0.5: 0.5), (ii) charcoal, brick pieces and sand (1:1:1) and (iii) soil and compost (1:1).

Thermocol/plastic pots were thoroughly washed with distilled water and dried to minimize the spread of disease or infections. Small holes were made in the pots to provide aeration and drainage of the water. The pots were filled $3/4^{th}$ with compost and watered as planting in the moistened compost is easier. The washed plantlets were

picked up with the help of forceps and the roots were carefully placed into the crevices of the compost. Single plantlet was potted in each pot. The pots along with the plantlets were covered with perforated polythene bags and were carefully sprayed with water and shifted to the glass house for hardening of the plantlets. The minimum and maximum temperatures of the glass house at the time of transplantation were 18°C and 32°C respectively. The relative humidity of the glass house was around 70 - 80%. The plantlets were watered in the evening on alternate days and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month. Readings were recorded after 60 days of hardening and subsequently the plantlets were made ready for field transfer.

RESULTS

Of the various compost used, the combination of soil and compost in 1: 1 ratio was found to be the best substratum for the survival and healthy growth of both *M. spathulata* and *M. wengeri*. Around 90.7% of *M. spathulata* (Table 3.1; Fig. 7 A) and 84% *M. wengeri* (Table 3.2; Fig. 7 C) survived within 4 weeks of transfer to pots containing soil and compost in equal ratio. However, the survival percentage of the plantlets in all the different compost tried was lower. On the other hand, the height of the plantlets transferred to pots differed significantly in the various composts. The plantlets of *M. spathulata* and *M. wengeri* attained a maximum height of around 8.9cm and 4.4cm respectively in the substratum containing soil and compost. A significantly increased height of 8cm was attained for the plantlets of *M. wengeri* in soil, sand and compost mixtures. Complete healthy green plantlets of both *M.*

Table 3.1: Hardening and acclimatization of *M. spathulata* plantlets

Treatment	Survival* (%)	Height (cm)*
Soil, sand and compost (dry cow dung) (1: 0.5: 0.5)	84.1±4.6	6.7±0.4
Charcoal, brick pieces and sand (1:1:1)	76.0±6.1	7.6±0.6
Soil and compost (1:1)	90.7±2.7	8.9±0.8
	NS	NS

'*' indicates mean average values of three repeated experiments with standard error (±SE)
NS: Non significant
ANOVA at 5% level of significance shows that all the parameters of growth are non-significant
Data recorded after 60 days

Table 3.2: Hardening and acclimatization of *M. wengeri* plantlets

Treatment	Survival * (%)	Height* (cm)
Soil, sand and compost (dry cow dung) (1: 0.5: 0.5)	80±2.3	8.0±0.3[b]
Charcoal, brick pieces and sand (1:1:1)	74.7±8.1	5.3±0.4[a]
Soil and compost (1:1)	84±8.3	4.4±0.5[a]
	NS	

'*' indicates mean average values of three repeated experiments with standard error (±SE)
NS: Non significant
ANOVA at 5% level of significance shows that all the parameters of growth are highly significant. Means followed by the same letters (a, b, c) are not significantly different according to Turkey's test ($p = 0.05$)
Data recorded after 60 days

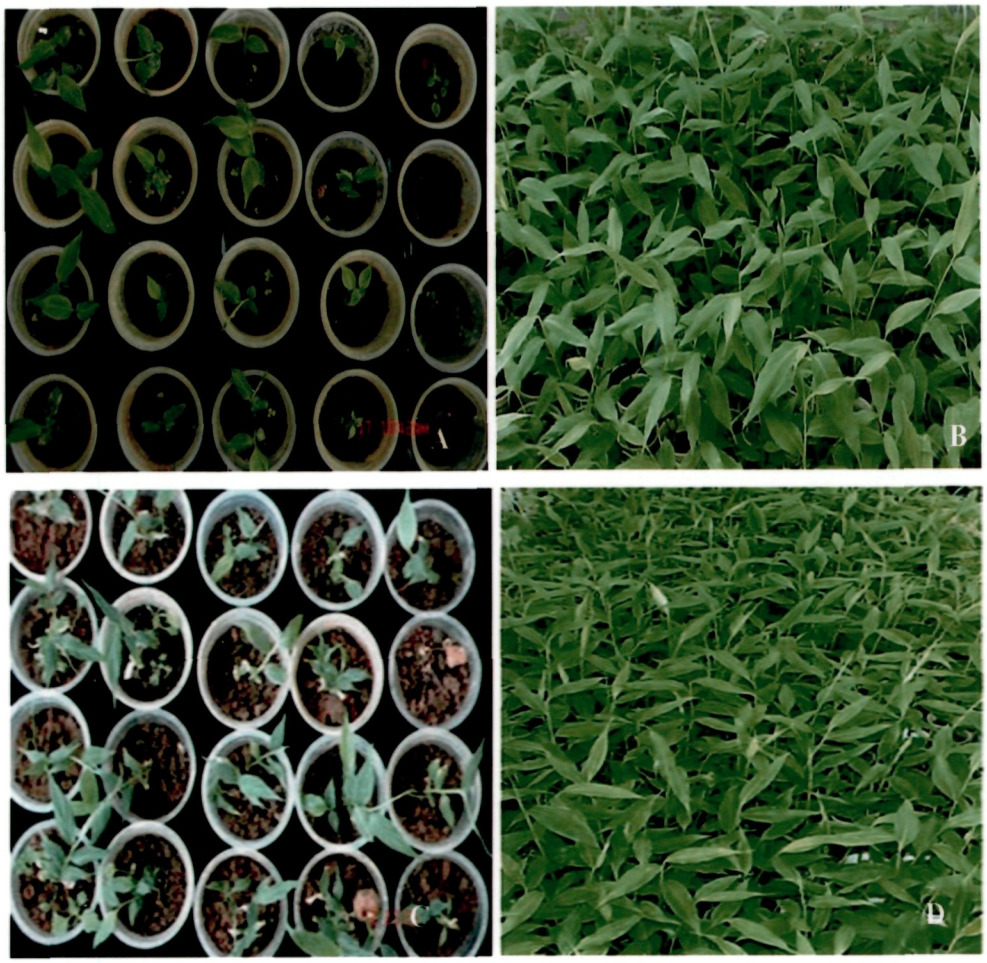


Fig. 7

spathulata and *M. wengeri* got established well in the substratum within 60 days of transfer (Fig. 7 B, D).

DISCUSSION

Successful hardening and acclimatization depends on suitable size of the plantlets and their state of growth *in vitro*. The type of plant materials viz. herbaceous, aquatic or woody, epiphytic etc. also determines the successful transfer of plant materials. Most of the orchids often require suitable substratum for higher survivability and non availability of suitable growth conditions may drastically decrease the survival percentage of transferred plantlets (Yadav *et al.*, 1988; Cribb, 1990; Robbins and Bell, 1990). However, most of the zingibers get efficiently hardened in normal soil and sand mixtures after transferring to the normal environmental conditions (Borthakur *et al.*, 1999; Koul *et al.*, 2005). Hardening in soil and sand at equal ratios resulted in 85% survivability of *Hedychium spicatum* (Koul *et al.*, 2005). Similarly, more than 80% of the transferred plantlets of *Alpinia galanga* survived in the potted 1:1 ratio of soil and sand mixture (Borthakur *et al.*, 1999). Also, a very high survivability and better growth of transferred plantlets of Zingiberaceae plants like *Curcuma zedoaria* and *Zingiber zerumbet* has been reported (Stanly and Keng, 2007). Besides soil and sand, addition of farm-yard manure to the substratum was found to enhance the acclimatization of *Zingiber officinale* (Sharma and Singh, 1997) and *Kaempferia galanga* (Shirin *et al.*, 2000). In the present study, the mixture of soil and sand in equal ratios resulted in very high percentage of survivability and healthier growth of plantlets of both *M. spathulata* and *M. wengeri*. Profuse rooting from the rhizomes allowed better adherence and efficient nutrient

uptake from the soil leading to healthier growth of the plantlets. Survival of transferred plantlets depends on their ability to carry out photosynthesis and withstand water loss. *In vitro* plantlets have the characteristics of less or no photosynthetic pigment, malfunctioning of stomata and marked decreased in epicuticular waxes that leads to the death of transplanted plants (Bhojwani and Dhawan, 1989). 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). Supply of diluted nutrient medium during hardening has been found to be beneficial for healthy growth of many orchids (Kumaria and Tandon, 1994). As the plants are perennial herbs the appropriate natural temperature and humidity during monsoon also favoured the higher survivability of the plantlets during post hardening period.

CHAPTER IV: ASSESSMENT OF GENETIC FIDELITY OF THE *IN VITRO* RAISED HARDENED PLANTLETS OF *MANTISIA SPATHULATA* AND *MANTISIA WENGERI*

INTRODUCTION

The asexual development of plants under *in vitro* conditions very often gives rise to somaclonal variations (Larkin and Scowcroft, 1981) which are often heritable (Breiman *et al.*, 1987). There are many reports which claim that useful morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al.*, 1989). The variations may be due to several factors, such as genotype used, pathways of regeneration and parameters employed (gross morphology and cytology) for assessing the effect of *in vitro* cultures (Swedlund and Vasil, 1985; Breiman *et al.*, 1987; Vasil, 1987, 1988). The variants associated with *in vitro* plant propagation may be classified into four different groups viz. physical and morphological changes in undifferentiated callus, variability in organogenesis, changes manifested in differentiated plantlets and chromosomal changes (Skirvin, 1978). A genetic level variation was first reported in tobacco cells culture by Murashige and Nakano (1967) who found that the frequency of aneuploid cells increased after 1 to 6 years. Where micropropagation is on a large scale, the prime concern is the maintenance of true-to-type nature of the offsprings. This can be achieved by developing a protocol based either upon axillary branching or somatic embryogenesis. These two methods are believed to give rise to true-to-type

plants since the organized meristems do not undergo genetic changes that might arise during cell division or differentiation from callus cultures (Shenoy and Vasil, 1992). The uncontrolled production of plants which are not true-to-type continue to pose problems in further use of the micropropagation protocols for large scale production of plants. Thus, for maintaining only true-to-type plantlets without any variants, techniques have been developed to test the fidelity of *in vitro* regenerants. DNA markers are more attractive means for examining somaclonal variations as they are not developmentally regulated. The molecular markers used for checking the fidelity of *in vitro* regenerants includes RFLPs, RAPDs, AFLPs, Simple Sequence Repeats (SSR), Sequence Tagged Microsatellite Sites (STMS), Sequence Tag Sites (STS), DNA Amplification Fingerprinting (DAF) and Microsatellite Primed-PCR (MP-PCR) (Mohan *et al.*, 1997). A number of reports have discussed the use of RAPD markers to study the polymorphism in populations (Jayanthi and Mandal, 2001), cultivars (Das nee Pal and Raychaudhury 2003) and even in clones (Singh *et al.*, 2004). RAPDs can circumvent some of the problems associated with other techniques such as RFLP and AFLPs analyses and are suitable to the genetic analyses of rare and endangered organisms (Bartish and Nybom, 1999; Fu *et al.*, 2003). Genetic stability of *in vitro* raised gingers has been assessed using RAPD markers (Rout *et al.*, 1998) that was developed individually by two groups viz. Williams *et al.* (1990), and Welsh and McClelland (1990). This technique is simple, quick to perform and requires only a small amount of DNA. A single short oligonucleotide primer, which binds to many different loci is used to amplify random sequences from a complex DNA template, such as plant genome. In a reaction of RAPD a single fragment of primer binds to genomic DNA at two different sites on opposite strands of the DNA template. The

presence of each amplification product intensifies complete or partial nucleotide sequence homology, between the genomic DNA and the oligonucleotide primer at each end of amplified product. Thus, this technique does not require DNA sequence information (Tingey and Tufo, 1993) and hence can been used for rapid analysis of the genetic fidelity of the *in vitro* raised plantlets of both *M. spathulata* and *M. wengeri*.

MATERIALS AND METHODS

For RAPD analysis, five 6 - month old hardened plantlets were randomly selected and compared for genetic similarity with mother stock of both *M. spathulata* and *M. wengeri*. For both the species, DNA was extracted from 100 mg of fresh leaves using DNeasy Plant Mini Kit (QUAGEN). The extracted DNA was finally dissolved in 100µl of AE buffer which yielded total DNA of around 50ng/µl. DNA (1µl) was loaded in 1% (w/v) agarose gel and electrophoresed under 60V constant power supply for 3h and the amplification products were visualized in Geldoc (UVP biodoc-IT) system. In a pre-screen with 30 primers based on amplification of mother plant, 5 arbitrary decamer primers (Operon Technologies, USA) produced easily scorable distinct amplification profiles that were reproducible and hence these were selected for polymerase chain reaction (PCR). PCR was performed in a reaction volume of 25µl containing ~ 50ng template DNA, 1X Taq buffer A containing 1.5mM $MgCl_2$, 60µM of each dNTPs, 0.05 units *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.) and 0.6ng primers (Operon Technologies, USA). Amplification was performed in a programmed thermal cycler (2700) supplied by Applied Biosystems (USA). After initial denaturation at 92°C for 3.30min, PCR was operated for 45

cycles consisting of a denaturing step (1.0min), 35°C primer annealing step (1.30min) and 72°C amplification (2.0min) step, and at the end of the run final amplification period of 7.0min (72°C) was appended. Amplification products were separated in 1.5% agarose gels in 1X TBE buffers stained with ethidium bromide and photographed with UVP biodoc-IT system. The electrophoretogram was scored for the presence of a band (1) or its absence (0). The data were analyzed using the SIMQUAL (similarity for qualitative data) routine to generate Jaccard's similarity coefficient. These similarity coefficients were used to generate dendrograms using Unweighted Pair Group Methods with Arithmetic averages (UPGMA) employing the Sequential Agglomerative Hierarchic Non-overlapping (SAHN) programmes from NTSys PC version 2.02 k software. Similarity matrix was compared with dendrogram using MxComp and correlation coefficient of the association was obtained to determine the significance level.

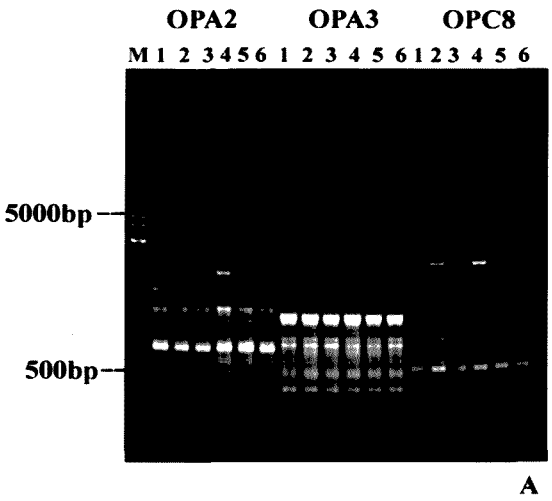
RESULTS

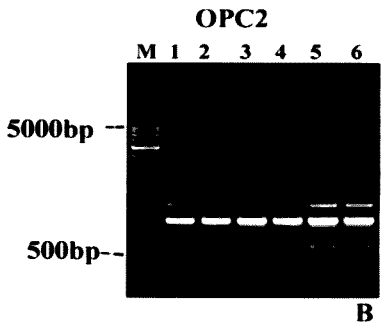
Size of bands produced by the primers ranged from 500 base pair with primers OPA2, OPA3, OPC2, OPC8 and OPC15 to 3250 base pair with primer OPA2 for *M. spathulata* (Fig. 8). On the other hand, the size of bands produced by the same primers ranged from ~800 base pair with primers OPC15 to ~3000 base pair with primer OPA2, OPA3, OPC2, OPC8 and OPC15 for *M. wengeri* (Fig. 9). From the analysis of the data the test for association using MxComp yielded the matrix correlation coefficient (r) as 0.917 and 0.778 for *M. spathulata* and *M. wengeri* respectively which was highly significant. From SIMQUAL generated matrix data it was observed that similarity coefficient ranged between 0.85 - 0.98 among

Table 4.1: Similarity coefficient among mother plant and *in vitro* raised hardened plantlets of *M. spathulata* and *M. wengeri* based on RAPD markers

	In vitro plantlets					
	1 *	2	3	4	5	6
(*M. spathulata*)						
1*	1.00					
2	0.91	1.00				
3	0.85	0.89	1.00			
4	0.89	0.98	0.91	1.00		
5	0.87	0.96	0.93	0.98	1.00	
6	0.87	0.96	0.93	0.98	0.96	1.00
(*M. wengeri*)						
1*	1.00					
2	0.93	1.00				
3	0.98	0.91	1.00			
4	1.00	0.93	0.98	1.00		
5	0.90	0.83	0.88	0.90	1.00	
6	0.95	0.88	0.93	0.95	0.95	1.00

1*- Mother plant


OPA2
OPA3
OPC8
M 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
5000bp
500bp
A


OPC2
M 1 2 3 4 5 6
5000bp
500bp
B

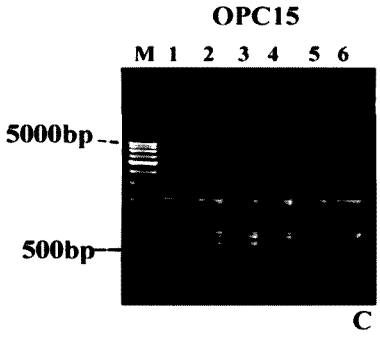
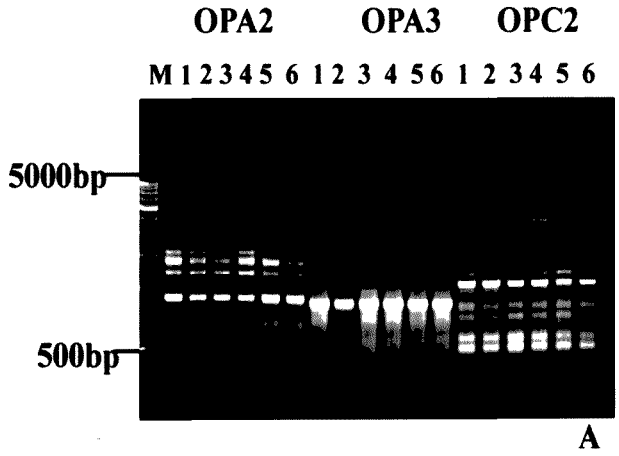

OPC15
M 1 2 3 4 5 6
5000bp
500bp
C

Fig. 8


OPA2
OPA3
OPC2
M 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
5000bp
500bp
A

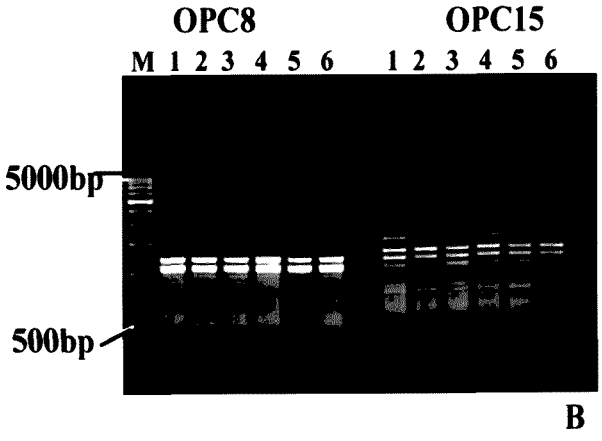

OPC8
OPC15
M 1 2 3 4 5 6 1 2 3 4 5 6
5000bp
500bp
B

Fig. 9

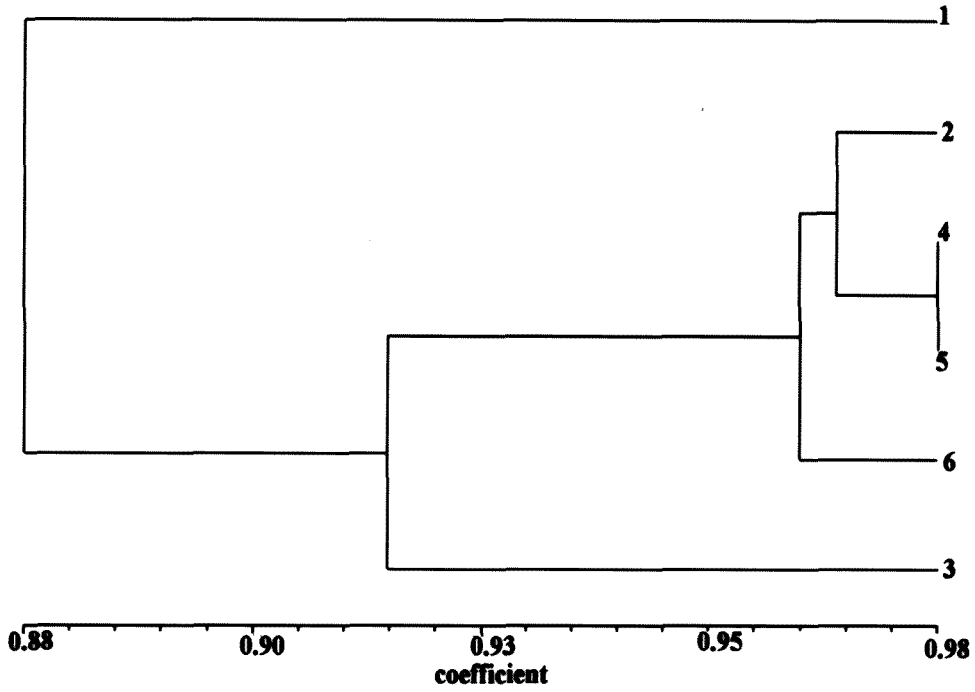

1
2
4
5
6
3
0.88
0.90
0.93
0.95
0.98
coefficient

Fig. 10

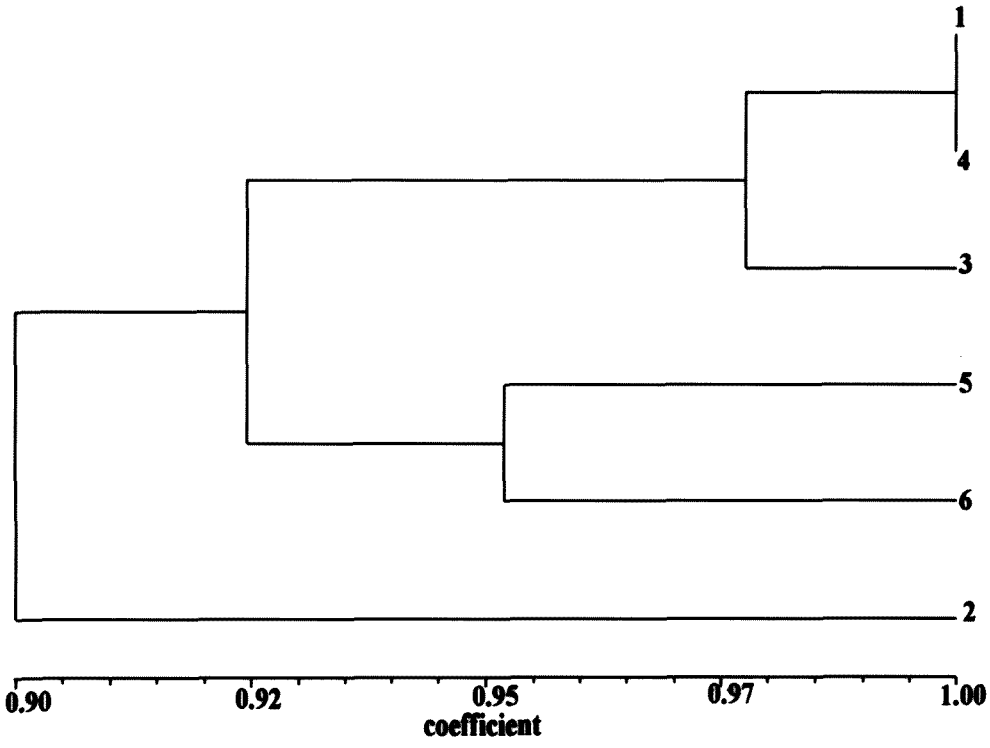

1
4
3
5
6
2
0.90
0.92
0.95
0.97
1.00
coefficient

Fig. 11

regenerated plants of *M. spathulata* and 0.83 - 0.98 among regenerated plants of *M. wengeri* (Table 4.1). From the matrix generated dendrogram it was amply clear that all the *in vitro* raised plants of both *M. spathulata* and *M. wengeri* had shown distant similarities with mother plant stock (Fig.10, Fig. 11).

DISCUSSION

Plants regenerated through tissue culture mostly come across the phenomenon of somaclonal variations which occupy a unique position because these have both an advantageous and a disadvantageous aspect. The main factors influencing somaclonal variations generated from tissue cultures are: (i) the degree of departure from organized growth, (ii) the genotype, (iii) growth regulators, and (iv) tissue sources (Karp, 1995). Although *in vitro* regenerants are expected to be the clones of their mother stock, it is however not always possible in many plants (Rani and Raina, 2000) and therefore analyzing the extent of genetic similarity of *in vitro* regenerants with their mother stock determines the possibility of transferring true-to-type plantlets to the field. Around 84% similarities have been reported in micropropagated plants of *Populus deltoides* (Rani *et al.*, 1995). Similarly, there are reports of genetic similarity in the range of 84 - 97% in field transferred plants of *Angelica acutiloba* (Watanable *et al.*, 1998) and 86% - 96% in shoot bud regenerated micropropagated plants of *Robinia pseudoacacia* (Kaushal and Kanwar, 2003). In the present study, the estimations of about 88% and 90% similarity for *M. spathulata* and *M. wengeri* respectively with their mother plant are near to the acceptable level of genotypic stability reported for field transferred plantlets of other species. By this parameter the regenerated plants presently produced for both *M. spathulata* and *M. wengeri* can be

categorized as normal plants and equivalent to mother stock with regard to genetic make up.

Genetic variations under *in situ* conditions in most of the zingibers including *Mantisia* are sluggish due to the clonal propagation through rhizomes (Nadgauda *et al.*, 1978) and lack of seed propagation (Sajina *et al.*, 1997; Miceli *et al.*, 2008). In this regard, minor genetic variations arising out of *in vitro* cultures without marked phenotypic alterations are expected to be beneficial for faster adaptability under varied climatic conditions and prolonged sustainability under biotic and abiotic stresses.

CHAPTER V: REINTRODUCTION OF THE HARDENED PLANTLETS OF *MANTISIA SPATHULATA* AND *MANTISIA WENGERI* FOR CONSERVATION IN EXPERIMENTAL GARDEN AND NATURAL HABITATS

INTRODUCTION

The North-eastern India, which is one of the biological hotspot of the world, is known for enormous rare plant resources exclusively endemic to this region. However, a great number of plant species including several unique and irreplaceable varieties are becoming extinct. Though the rarity is a likely factor in the survival of species it is not the prime cause of the massive rate at which species are becoming endangered and extinct. The principal factor is the extensive damage to or destruction of the habitats of vast number of species, especially in the species-rich tropics. The preservation of species *in situ* is of primary importance for maintaining the broadest range of plant diversity. *In situ* preservation allows natural ability of the endangered plants to withstand the natural selection thereby increasing the survivability through interacting with other life forms in the habitat. Thus, *in situ* conservation has the potential for long-term preservation of communities and populations, under conditions of continuing adaptation (Pence, 1999). However, in certain cases the extent of destruction of natural habitat of plants becomes so severe that only few representatives of the plants sustains. In such cases, *ex situ* preservation can play role

in backing up taxa which are particularly threatened or are rare in the wild. The maintenance and propagation of species in Botanical gardens and arboreta as well as in seed and spore banks have provided a valuable safeguard against loss for many rare species (Laliberte, 1997). The development of a reliable *in vitro* protocol is of great importance for conservation of rare plant species (Bramwell, 1990). The threatened plants could be multiplicated through tissue culture and exposed to *ex situ* collections to offset the pressure on the natural populations as well as for medicinal and ornamental purposes. *Ex situ* conservation provides the freedom to select individual species for preservation. Botanical gardens can make distinct contribution of endangered species in either of the two ways (Pence, 1999).

1. Assemble information on endangered species, their geographical distribution, diversity, degree of rarity, endangered state, and their representation in Botanical gardens or other collections.
2. Assemble and maintain representative samples of the genetic diversity of endangered species, to safeguard their continuing survival and to serve as a readily available resource for research and education, for reintroduction into natural communities and for distribution to industries.

Reintroduction demands a good deal of management, skill and resources for operations extending from collecting of planting materials (usually seeds), raising of seedlings to the choice and preparation of transplant sites, planting, protecting and further safeguarding of transplant populations. Information on causes of or circumstances contributing to a decline of the original population would be of interest for attempts to counter adverse ecological factors, especially in the seedling or juvenile stage. These may include the presence of predators, parasites, competitors,

nutritional imbalances or decline or absence of beneficial co-occurring species such as pollinators or seed dispersers or of an adequate water supply. Thus, developing a model for species distribution using environmental surrogates of known locations can provide a range of its potential distribution for conservation planning (Giriraj *et al.*, 2008). Inferential procedures that provide robust and reliable predictions of geographic distribution and ecological conditions of species are thus critical for *in situ* and *ex situ* reintroduction of endangered species for their effective conservation. This approach has recently been explored under the rubric of 'ecological niche modelling' (ENM), and refers to reconstruction of ecological requirements of species (Peterson 2001).

A number of endangered plant species have been re-established in their natural habitats using *in vitro* propagation methods in the past (Tandon *et al.*, 1990; Tandon and Rathore, 1992; Seeni and Sabu, 1997). In recent years, a large number of plants have been conserved and reintroduced into natural habitats. *Spiranthes brevilabris* Lindley (Orchidaceae), appeared in 1999 to be restricted to a single population in Levy County, Florida. That population consisted of 152 plants. After *in vitro* seed germination the plantlets were reintroduced into six different sites in Florida with 100% survival (Stewart *et al.*, 2003). Similarly, it appears that the long-term viability of wild populations of *Nepenthes clipeata* is low. Therefore, it is imperative that *ex situ* conservation measures be implemented. The *Nepenthes clipeata* Survival Project (*Nc*SP), under the auspices of The International Carnivorous Plant Society, has been devised to maximize the genetic diversity of this species in cultivation (Cantley *et al.*, 2004). Reports on eco-rehabilitation of the endangered blue Vanda into alien forest habitats at Ponmudi and Palode in southern ranges of the Western Ghats has been

achieved with 70 - 80% success (Seeni and Latha, 2004). *Aerides crispum* is one of the most important orchids, valued for its beautiful inflorescence/flowers. This species is endemic to South India and its natural populations are dwindling due to over exploitation (Rao, 1998). Well acclimatized plants were reintroduced into alien forest habitats (Murthy, 2005). *In vitro* raised field transferred plants of *Rhododendron poniticum* survived to an extent of 87% after reintroduction into their natural habitats within eight months (Almeida *et al.*, 2005). Among zingibers micropropagated plants of *Hedychium spicatum* have been successfully reintroduced in the natural habitat in recent years (Koul *et al.*, 2005). Besides, the species of *Mantisia* available in Mizoram, India, there are many more medicinally important endemic zingibers of this region such as *Rhynchanthus longiflorus, Alpinia calcarata, Curcuma aeruginosa, C. caesia, Hedychium spicatum, Kaempferia galanga, K. rotunda, Zingiber zerumbet,* etc. (www.universityofcalicut.info; Prasanthkumar *et al.*, 2005) which are on the verge of extinction and require due attention for recovery and reintroduction in natural habitats.

MATERIALS AND METHODS

One-year old healthy green hardened plantlets of both *M. spathulata* and *M. wengeri* were transferred to the natural habitats for rehabilitation during the month of June 2008. Around 100 plants of *M. spathulata* were planted in the soil of rocky hills along the road side of village Lunglawn at an elevation of 1183m at Lunglei district, Mizoram. Similarly, 100 plants of *M. wengeri* were planted in the soil of rocky hills along the road side of village Sethlun at an elevation of 1395m, Lunglei district, Mizoram.

As the natural habitats of both the species in Mizoram remains under the threat of frequent landslides and slope failurs, effort was under taken to transfer the plantlets in the Experimental Garden at Shillong at an altitude of 1370m above mean sea level.

Around 500 hardened plantlets of *M. spathulata* and *M. wengeri* were planted in the soil of Experimental Garden, Plant Biotechnology Laboratory, Department of Botany, North Eastern Hill University, Shillong, Meghalaya during the month of October, 2006. The plants shed their leaves by the end of November but the rhizomes remained dormant in the soil till the next pre-monsoon season.

All the plantlets were watered for a week to establish them in the soil. Later, the natural rainwater of monsoon irrigated the plantlets under the climatic conditions of Shillong. The sloppy hills of the transplanted sites prevented water logging and maintained proper drainage of the soil which is an important requisite for *Mantisia* plants.

RESULTS

Around 52% and 45% hardened plantlets of *M. spathulata* (Fig.12 A, B) and *M. wengeri* (Fig.12 C, D) survived under natural climatic conditions of Lunglei, Mizoram after reintroduction. Around 80% dormant rhizomes of *M. spathulata* flowered during the month of April, 2007 in the experimental garden (Fig.13 A). More than 90% of the dormant rhizomes of both *M. spathulata* and *M. wengeri* transferred to experimental garden sprouted 3 - 5 vegetative shoots immediately after the initial rainfall during the month of May, 2007 (Fig.13 B, C & D). Sprouting of shoots continued till the month of July, 2007 and attained an approximate height of 15 - 20 inches till October. From preliminary observation, marked phenotypic variations

Fig 12. Reintroduced plantlets of *Mantisia spathulata* and *Mantisia wengeri* in natural habitats of Lunglei, Mizoram

(A) Hardened plantlets of *M. spathulata* for field transfer

(B) Plantlets of *M. spathulata* transferred in the soil of natural habitat

(C) Hardened plantlets of *M. wengeri* for field transfer

(D) Plantlets of *M. wengeri* transferred in the soil of natural habitat

Fig. 12

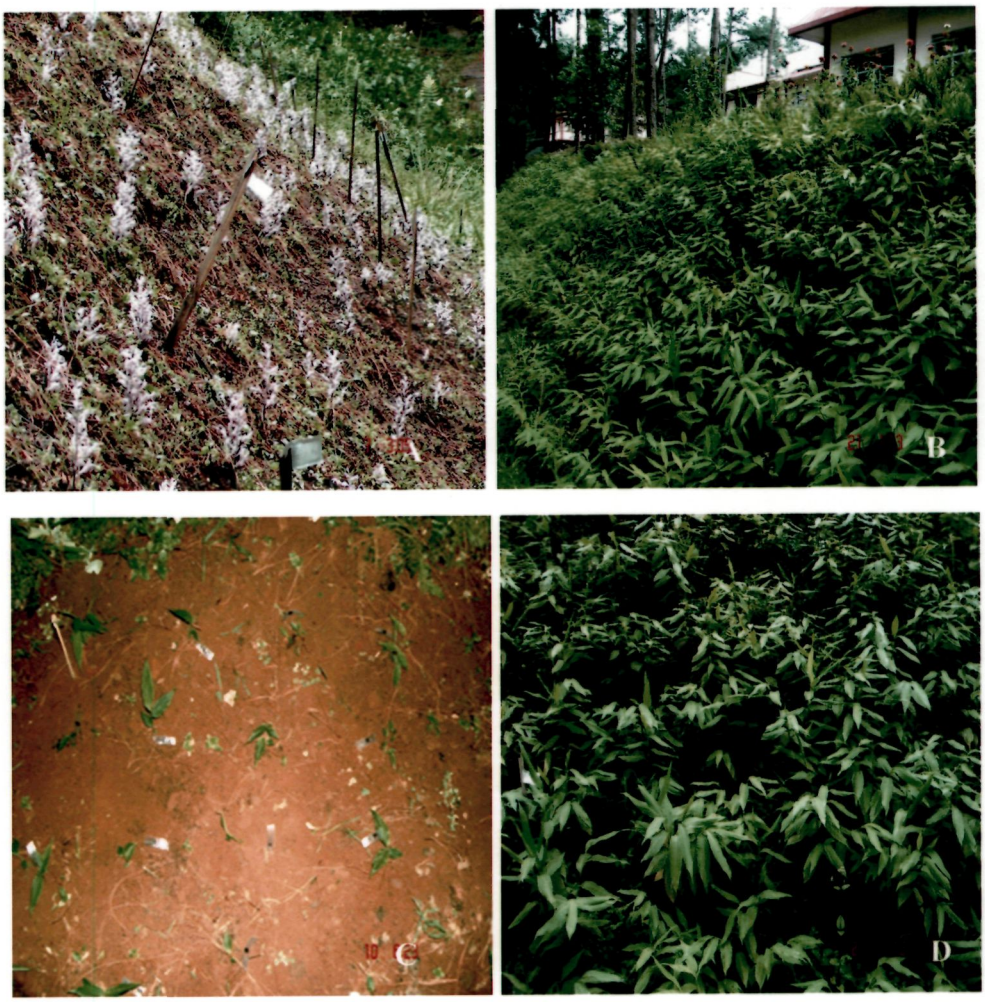


Fig. 13

were not noticed in any of the plantlets introduced so far in the garden. The blooming of large number of flowers in the experimental garden attracted various insect pollinators towards these flowers resulting in the pollination of most the flowers within one month. This led to the formations of numerous seed bearing capsules that matured under the field conditions. The mature capsules (3 - 4 weeks old) dehisced soon after the seed set and got dispersed in the soil. This phenomenon was however, not recorded in the plants grown under glass house conditions.

All the plantlets shed their leaves by the end of November and the rhizome became dormant in the soil. No new plantlets from the dispersed seeds were seen to grow in the area of transplanted sites of the experimental garden till the end of November.

DISCUSSION

In vitro-raised hardened plantlets of both the species reintroduced in their respective habitats at Lunglei have shown limited success with only 52% and 45% survivability for *M. spathulata* and *M. wengeri* respectively. Plantlets reintroduced in the natural habitats come across various factors that hinder their adaptation in natural habitats. Factors such as competition with other organisms and plant species in the wild habitat, heavy rainfall, landslides, predation, diseases, etc. prevailing in the natural habitats become responsible for the lower survivability of the plantlets in the natural habitats.

The Lunglawn-Sethlun area of Lunglei, Mizoram is prone to landslides or slope failures and therefore cause loss of human lives and properties almost every year besides disrupting communication links. The landslides and other slope failures

phenomena occur in the mountainous terrains due to combined effect of a number of geological factors such as slopes, rock and soil types and anthropogenic activities. The rock type of the region belongs to the Surma group comprising Bhuban and Bokabil formations (Sarkar and Nandy, 1974; Ganguly, 1975; Dasgupta, 1984; Nandy, 2001). These formations are composed of shale and silt with intercalations and infiltrated water flows through the contact zone of such rock formations. The thick soil cover present in the study area is predominantly sandy clayey soil mixed with deeply weathered fragile, slumped, soft, splintery shale and silt stones with slope wash soil. The safety factor of the soil falls conspicuously with gradual saturation and has poor shear strength. High pore pressure during rainy season with zones of waterlogged muds, detrimental thin sandstone beds and the hydrostatic pressure lead to an increase in the slope failure during monsoon season. Hence, the herbaceous plants having the root depth to a maximum of 0.5m are washed off along with the slides during monsoon.

From the present investigation of the natural habitats, it was observed that the whole area is under slides and subsidence. The sliding activity aggravates during rainy season and has direct impact on the flowering and seed formation of *M. spathulata* and *M. wengeri* which occurs during this season. Thus, it is suggested that the restoration and rehabilitation of the collapsing habitats itself must be of prime concern before further trial on reintroduction of plantlets is made in these habitats in future.

The plantlets introduced in the experimental garden are carefully nurtured and therefore, do not come across any of these limiting factors. Thus, the success rate of survivability of plantlets is more in experimental garden as compared to the plantlets

that had been reintroduced in the natural habitats. Since the tissue cultured-raised plantlets were successfully established in the soil under the natural climatic conditions of Shillong, it might be concluded that most of the agro-climatic factors such as altitude, precipitation, temperature, humidity, light intensity, rainfall, soil pH etc. prevailing in the natural habitats of Lunglei, are similar to a great extent with the conditions prevailing in Shillong. Therefore, similar climatic conditions helped in the adaptation of these two species of *Mantisia* in the new habitat of Shillong. Analyzing the ecological parameters required for the growth of these species using niche ecological modeling tools would further allow the reintroduction of these species at different places besides their natural habitats.

It is inferred from the study site that the depletion of *M. spathulata* and *M. wengeri* from natural habitats is mainly due to excessive soil erosion, landslides and failure of seed germination in natural soil. Any natural calamity would totally wipe out the remaining countable plants from their natural habitats in future. Hence, remedial measures such as immediate afforestation of the barren hillslopes with fast growing fibrous rooted plants such as *Musa* and *Bromus* species and trees like *Eucalyptus*; construction of horizontal and vertical drainage for draining excess water from slopes; filling up of subsidence cracks with sand mixed coaltar and lined drains for waste water disposal etc., must be done so that the plant diversity in their natural habitats is retained (Nandy, 2001). Besides these measures, as a strategy we suggest the introduction of these species of *Mantisia* in Meghalaya to ensure its survival subsistence.

The reintroduction of the plants in the experimental garden has circumvented the problem associated with its geographical isolation and seasonal barriers. As it is

possible to monitor the plants easily in the experimental garden, studies on reproductive behaviour, molecular characterization, medicinal properties etc. can be easily carried out for these two species of *Mantisia*.

CHAPTER VI: SEED GERMINATION OF *MANTISIA SPATHULATA* AND *MANTISIA WENGERI* FOR CONSERVATION OF GENETIC VARIABILITY

INTRODUCTION

Seed germination is defined as the protrusion of embryonic axis from the seeds to resume plant growth. The process involves considerable amount of metabolic activities and are regulated by various factors mainly genotype of the plant and physical factors e.g. light intensity, temperature, humidity, growth regulators, soil conditions etc. The possibility of sustaining the genetic diversity of rare and endangered plants becomes possible through seed propagation (Pence 1999, Tandon and Kumaria, 2005). Under *in situ* conditions genetic variations in most of the zingibers are sluggish due to vegetative propagation through rhizomes (Nadgauda *et al.*, 1978) and lack of seed propagation (Sajina *et al.*, 1997; Miceli *et al.*, 2008). Although, seeds possess genetic diversity, the natural propagation of many rare and endangered plants are limited due to many factors for e.g. seed dormancy in *Nepenthes khasiana* (Rathore *et al.*, 1991), poor seed viability in *Rauvolfia micrantha* (Sudha and Seeni, 1996). In many others species such as *Delphinium malabaricium* and *Paronychia chartacea*, little or no seed is produced (Agrawal *et al.*, 1991; McKently and Adams, 1994). In some cases, seeds have particular requirements for germination such as *Pholisma sonorae*, an endangered parasitic plant requires host root tissues for germination (Pence, 1999). Similarly, mycorrhizal association with

particular fungal species is necessary for early stages of development in orchids because of which less than 5% of the orchid seeds germinate in nature (Rao, 1977). When seeds are available the germination of seeds under *in vivo* conditions has been studied for many endangered plants. The study on the seed germination of 10 elite species of *Rhododendron* under *in vivo* conditions in various soil treatments viz. rhizosphere soil, non-forest soil and sterilized soil in various combinations indicated the presence of certain factors mainly of microbial origin that highly influences the seed germination (Singh *et al.*, 2008). Attempt was made to test the viability of the seeds of *Phyllanthus beddomie* by sowing them in fertile soil (Maridass and Thangavel, 2008). However, the germination recorded was very poor and therefore *in vitro* germination of *Phyllanthus beddomie* was carried out using growth regulators in the medium. Hence, when seeds are unable to germinate in soil under natural conditions, *in vitro* germination remains the only viable option for the immediate large scale propagation, especially for rare and endangered plants.

The incorporation of growth regulators in the culture media greatly influences the promotion of seed germination in many plants including rare and endangered orchids (Sharma and Tandon, 1986; Van Waes and Debergh, 1986; Kumaria, 1991; Talukdar, 2001; Nagaraju *et al.*, 2003). The addition of GA_3 in the culture medium has been usually found to promote germination of seeds in large number of plants such as *Nepenthes khasiana* (Rathore *et al.*, 1991), *Aldrovanda vesiculosa* (Kondo *et al.*, 1997), *Primula glaucescens* (Cerabolini *et al.*, 2004) etc. In many plants, the capsules containing seeds dehisce immediately after seed set and the seeds get dispersed in the soil. Similar phenomenon is also observed in the two species of *Mantisia.* Since, the number of plants of the two species in their natural habitats have

reduced to a great extent it becomes necessary to study the viability of the seeds under *in vivo* conditions in the natural soil. Moreover, the seeds being heterogenous allow the production of large number of diverse seedlings which is highly desirable for these two endangered plant species of *Mantisia.*

MATERIALS AND METHODS

In vivo seed germination

The fruiting capsules enclosing seeds were collected from the nature and stored in refrigerator (4°C) until the experiments were initiated. To study *in vivo* seed germination, soil was collected from the natural habitats of both the plants during early monsoon season and ground to fine particles. Similarly, soil collected from the experimental garden was maintained as control in the experiment. The pH of the soil was determined using pH meter (Eutech Instruments, UK). Seeds (~ 1.0 - 2.0 mm in size) were kept for germination in soil placed over water soaked filter papers in petridishes (12.5 x 12.5 cm) inside the plant growth chamber (Narrang Scientific Works Pvt. Ltd., New Delhi). These were regularly sprinkled with water every three days for maintaining optimum humidity (70 - 80% RH) under 12 h photoperiod with a PPFD of 40.5μmoles $m^{-2}s^{-1}$ at 25±2°C. The experiments were repeated thrice with 120 seeds per experiment and percentage of seed germination was recorded after 4 weeks of sowing. Same cultural practices were followed for both the species.

In vitro seed germination

To execute *in vitro* seed germination, one-week old immature capsules of *M. spathulata* and *M. wengeri* were treated with 0.2% cetramide (Shalaks Pharm Industrial Ltd., New Delhi) for 10min, and washed several times with sterile pure

water. Finally, the capsules were surface sterilized with 0.075% mercuric chloride for 5min and rinsed several times with sterile pure water. Ten seeds dissected out from the aseptic capsules were inoculated in 150ml conical flask containing 50ml of sterilized MS medium supplemented with 3% sucrose and GA_3 at various concentrations (1.4 - 28.9μM). MS medium without GA_3 was maintained as a control. The medium was solidified with 0.8% extra pure agar (Hi-media, India) and pH was adjusted to 5.8, prior to autoclaving for 15min at 1.06kg cm^{-2} (121 °C). The cultures were incubated at 25±2°C under 12h photoperiod with 40.5μmole$m^{-2}s^{-1}$ light intensity provided by cool white fluorescent lamps. Observation was made on the percentage of seed germination after 4 weeks of culture. The experiments were repeated thrice with ten replicates per treatment. Statistical analysis was done by ANOVA ($P<0.05$) and means compared using Turkeys test (software Origin 7.0).

RESULTS

The investigation on germination of seeds under *in vivo* conditions for both the species showed very less percentage of germination. A maximum of only 19.7% seeds of *M. spathulata* germinated within 4 weeks in the soil of natural habitat (Table 6.1; Fig. 14 A). The germination was further reduced in the control with only 10% seeds showing germination. Similarly, a maximum of 24.2% seed germination was recorded for *M. wengeri* within 4 weeks in the soil from natural habitat which was slightly higher as compared to control where only 8.3% germination was recorded (Table 6.1; Fig. 14 D). The pH of the soil from natural habitats and that of the control was found to be similar and acidic. Although the seeds showed very poor germination in the soil under *in vivo* conditions, the germination got significantly enhanced in MS medium

Table 6.1: Percentage of *in vivo* seed germination of *M. spathulata* and *M. wengeri* in soil

	Germination* (%) *M. spathulata*	Germination* (%) *M. wengeri*
Experimental garden soil (pH 6.4)	10±1.5[a]	8.3±1.5[a]
Natural soil (from habitat) *M. spathulata* - soil pH 6.7 *M. wengeri* - soil pH 6.8	19.7±0.7[b]	24.2±1.4[b]

'*' indicates mean average values of three repeated experiments with standard error (±SE)

ANOVA at 5% level of significance shows that *in vivo* seed germination of both species are highly significant. Means followed by the same letters (a, b) are not significantly different according to Turkey's test (p = 0.05)

Data recorded after 30 days

Table 6.2: Percentage of *in vitro* seed germination of *M. spathulata* in MS medium with GA_3

GA_3 (μM)	Germination* (%)
0.0	16.7 ± 3.3^{a}
1.4	53.3 ± 3.3^{b}
4.3	90.0 ± 5.7^{bc}
7.2	83.3 ± 6.7^{bc}
14.2	63.3 ± 3.3^{b}
28.9	36.7 ± 3.3^{a}

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that the germination of seed under *in vitro* conditions is highly significant. Means followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p=0.05)
Data recorded after 30 days

Table 6.3: Percentage of *in vitro* seed germination of *M. wengeri* in MS medium with GA_3

GA_3 (µM)	Germination* (%)
0.0	13.3±3.3[a]
1.4	30.0±5.7[a]
4.3	56.6±3.3[b]
7.2	96.6±3.3[bc]
14.2	73.3±3.3[b]
28.9	46.6±6.6[b]

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance shows that the germination of seed under *in vitro* conditions is highly significant. Means followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p=0.05)
Data recorded after 30 days

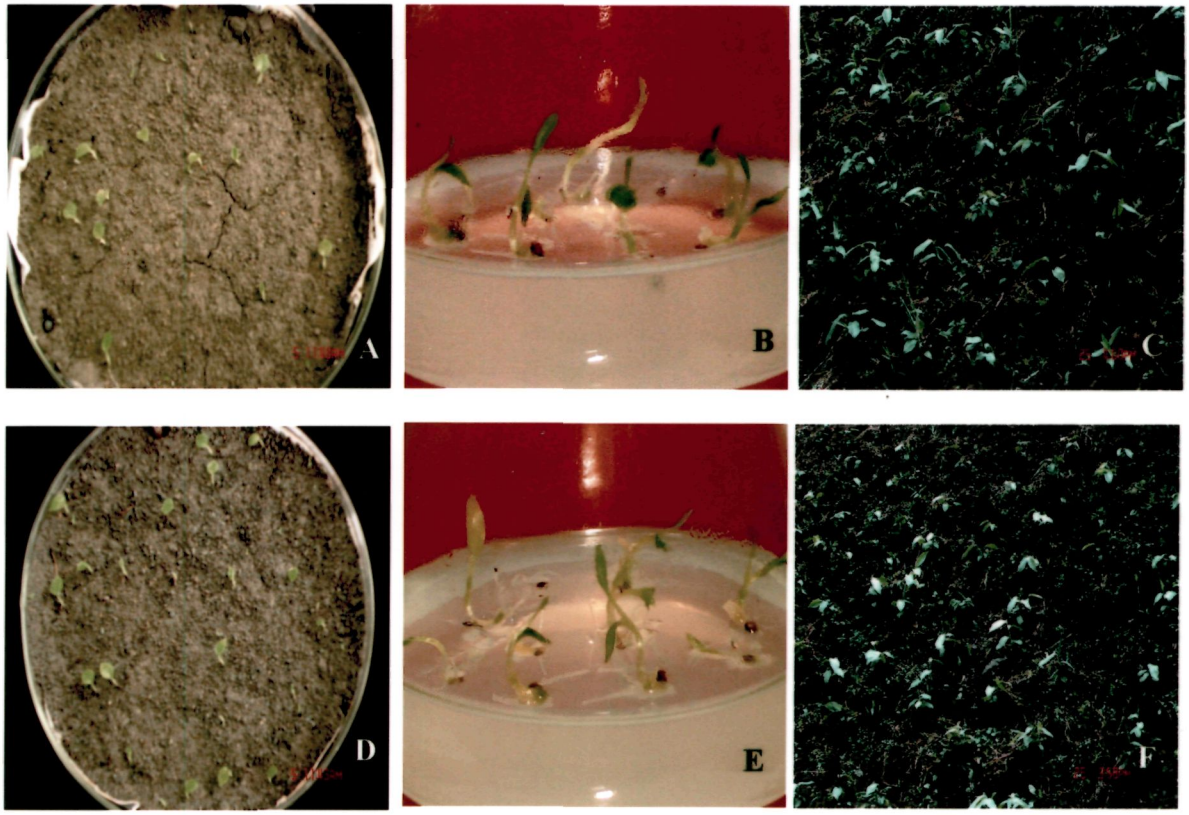


Fig. 14

incorporated with various concentrations of GA_3 under *in vitro* conditions. A maximum of 90% seeds of *M. spathulata* germinated in MS media supplemented with 4.3μM of GA_3 which was significantly higher as compared to the control (Table. 6.2: Fig. 14 B). The germination of seeds was observed to get reduced with either increase or decrease in the concentrations of GA_3 beyond this optimum level. Similarly, the enhancement of seed germination was also recorded for *M. wengeri* under *in vitro* conditions. A maximum of 96.7% seeds germination was recorded for *M. wengeri* in MS medium supplemented with 7.2μM of GA_3 which was significantly higher as compared to the control (Table 6.3; Fig. 14 E). The germination of seeds was observed to get reduced with either increase or decrease in the concentrations of GA_3 beyond this optimum level.

DISCUSSION

The fruiting capsules of *M. spathulata* and *M. wengeri* bearing numerous seeds were seen to dehisce immediately after seed formation. Since new young seedlings were not seen growing around the areas where the capsules dispersed, it could be assumed that the seeds fail to germinate in nature either due to abiotic (rainfall and landslides) or biotic factors viz., fungal contamination or infestation, seed dormancy, lack of symbiotic associations etc. as has been reported in many other instances (Hosoki and Sagawa 1977; De lange *et al.*, 1987; Rathore *et al.*, 1991; Singh *et al.*, 2008). Several authors have showed that temporal variation in seed germination depends on hydration intensity, temperature regime, light conditions, as well as on ontogenic experience during dormancy release (Garvin and Meyers 2003; Walck and Hidayati, 2004; Zia and Khan 2004; Kagaya *et al.*, 2005). Therefore, in natural

conditions seedling emergence occurs sporadically only when environmental conditions necessary for a particular genotype are met. In addition, a number of seeds in each population of the particular species may be programmed to remain dormant even in suitable environmental conditions due to the existence of multiple-level dormancy (Garvin and Meyers, 2003).

A higher percentage of seed germination was recorded under *in vitro* conditions, in medium supplemented with optimum concentration of GA_3. This could be probably due to enhancement of amylase synthesis as a result of GA_3 treatment in the germinating seeds (Paleg 1960; Amen 1968; Galston and Davies, 1969). Seed germination was poor in the control and at higher and lower concentrations of GA_3. However, an optimum concentration of GA_3 stimulated higher seed germination for *M. spathulata* and *M. wengeri*. The regulation of seed germination with optimum level of GA_3 has been also reported in *Physoplexis comosa* and *Primula glaucescens* (Cerabolini *et al.*, 2004). The seeds of *Physoplexis comosa* germinated only under *in vitro* conditions, in which germination percentages were extremely low without GA_3 (1.7%). In this case, optimum GA_3 concentration of $100mgl^{-1}$ in the medium stimulated more than 90% of seed germination. However, the germination got reduced at very high concentrations of GA_3 (250 - $500mgl^{-1}$). The seeds of *Primula glaucescens* germinated in both sterile and non-sterile conditions with rapid and earlier germination in the presence of 10 - $500mgl^{-1}$ GA_3. In the present study, the application of GA_3 in the medium has been also found to greatly influence higher percentage of seed germination unlike the medium without GA_3. Reports on seed germination of Zingiberaceae species are very limited. However, Brain and Richard (1993) reported a maximum of only 35% seed germination of pink ginger (*Alpinia*

purpurata) and 25% germination in large cardamom (*Amomum subulatum*) in MS medium without growth regulators (Sajina *et al.*, 1997). Similarly, a highest of only 20% seed germination occurred in *Renealmia mexicana* within 40 days in basal MS medium (Miceli *et al.*, 2008). These reports shows that using only the basal medium without growth regulators cannot significantly affect the seed germination in most of the zingibers. Therefore, in the present study, the higher percentage of seed germination of the *Mantisia* species was recorded with the application of optimum concentration of GA_3 in the culture medium. *In vitro* raised seedlings measuring around 1 - 2cm in size, on being transferred to the MS basal medium without GA_3, produced well-developed plantlets with healthy roots and shoots within 3 weeks of subculture. To maintain a wide genetic basis it is preferred to establish cultures of rare and endangered plants from seeds (Benson *et al.*, 2000). Hence, the *in vitro*-raised seedlings obtained under *in vitro* and *in vivo* conditions of both the species were weaned in the glass-house and transferred to the experimental garden after hardening (Fig. 14 C, F).

CHAPTER VII: LONG-TERM CONSERVATION OF *MANTISIA SPATHULATA* AND *MANTISIA WENGERI* THROUGH CRYOPRESERVATION OF SEEDS

INTRODUCTION

As most of the rare and endangered plants of high value are getting extinct at an alarming rate due to disturbances of their natural habitats an additional method besides the *ex situ* conservation is the storage of germplasm under ultra low temperatures and dry conditions i.e. cryopreservation. Conservation through cryopreservation is considered to be one of the most useful and easy means for preserving genetic resources (Roos and Davidson, 1992).

Though numerous methods are available and used in cryopreservation studies, some new methods have been developed recently which are cheaper and easier to perform and avoids the use of toxic chemicals like DMSO. Encapsulation-dehydration is a method based on successive osmotic and evaporative dehydration of the tissues (Dereuddre *et al.*, 1990). Here the material which is normally a shoot tip/somatic or zygotic embryo is encapsulated in sodium alginate beads. These encapsulated beads can be precultured in high osmoticum mainly in high sucrose solution and then air dried and plunged in liquid nitrogen. Encapsulation-dehydration procedure has been successfully applied to many temperate and tropical species such as seeds of *Dactylorhiza fuchsia, Anacampsis morio* and *Vanda coerulea* (Wood *et al.*, 2000;

Jitsopakul and Thammasiri, 2005), seeds and protocorms of *Oncidium bifolium* (Flachsland *et al.*, 2006), etc. and shoot tips of *Dendrobium* Walter Oumae (Lurswijidjarus and Thammasiri, 2004).

Another most viable easy method for cryopreservation is the desiccation of plant material in sterile air or silica gel to minimum moisture content and then directly plunging the material in LN followed by quick rewarming and growth in regeneration medium. This has been successfully carried out in many plants. The embryonic axes of *Quercus ilex* were desiccated to 0.34g water/g dry weight with 80% survivability (Gonzalez-Benito *et al.*, 2002). Similarly embryos *of Zizania texana* (Walters *et al.*, 2002), *Sechium edule* (Esquivel and Engelmann, 2002), *Citrus madurensis* (Cho *et al.*, 2002 a) have been successfully cryopreserved through desiccation of axis to minimum moisture level. Cryopreservation trials on whole seeds can withstand more extreme dehydration than excised embryonic axes thus, there would be less water available for ice formation during freezing. Seeds are classified as being either desiccation tolerant (capable of retaining viable) being dried to virtually any moisture content and are termed as orthodox seeds, or sensitive (seeds losing viability after being dried below a critical limit usually 12 - 30% moisture) and are termed recalcitrant seeds. This classification is a great simplification and a number of intermediate types exists known as sub-orthodox seeds. The use of whole seeds would also avert the problems associated with cryopreservation of excised embryonic axes, such as development of appropriate embryo excision methods and *in vitro* culture techniques (Chin *et al.*, 1988). Furthermore, problems of contamination by fungi and bacteria observed in embryonic axes of non-orthodox seeds (Mycock and Berjak, 1990; Berjak, 1996) can be overcomed by cryopreservation of intact seeds. Most

desiccation-tolerant seeds survive cryogenic storage (Stanwood, 1985). Temperature less than about -130°C are desired for cryopreservation because of very low molecular kinetic energies, absence of liquid water and extremely slow diffusion. Water in seeds shows different characteristic depending on whether it is above or below the so called critical water content. The critical water content usually resides around 20% on fresh weight basis, varying from 15 - 25% depending on species. Water content lower than the critical value is called bound water, which is tightly bound to the components of seed cells. This type of water is not freezable even at ultra low temperatures. Water content above the critical value, on the other hand, is called free water, which is not bound to the cell components and freezable at extreme low temperature. Desiccated orthodox seeds, tolerating water content lower than the critical values, have no freezable water and can survive exposure to liquid nitrogen temperature (LN; -196°C). In contrast, recalcitrant seeds, even if partially dehydrated to the extent that there is no viability loss, still have free water in themselves and are unable to tolerate exposure to LN. Thus, for long-term storage of heterogenous seeds, cryopreservation through dehydration was undertaken for the species of *Mantisia*.

MATERIALS AND METHODS

Experimental material

The seeds from the unopened capsules (two weeks after seed set) of *M. spathulata* and *M. wengeri* were used for cryopreservation through dehydration. The capsules were surface sterilized as mentioned in Chapter VI and used for setting up the experiments for cryopreservation.

Dehydration of seeds

To protect injury against LN treatment and optimize the osmotolerance of seeds against dehydration, seeds after surface sterilization were precultured in liquid MS medium supplemented with various combinations of Sucrose (0.0 - 3M) and Glycerol (0.0 - 8M) at different time intervals (0.0 - 4h). The precultured seeds were then taken into the sterile laminar flow and gradually dehydrated over a period of time (0.0 - 8h). These dehydrated seeds were cultured on regrowth medium (germination medium optimized in Chapter VI) to observe the germination at different moisture levels. The moisture content of the seeds was determined by drying them in the oven at 102°C for 48h (Fabre and Dereuddre, 1990). The percentage of moisture content was expressed on fresh weight (FW) basis using the formula,

Moisture content (%) = (Fresh weight – Dry weight) ÷ Fresh weight x 100

Storage in Liquid Nitrogen (LN)

Around 10 dehydrated seeds were placed in each 2ml cryotube and then directly plunged into liquid nitrogen for 1h. The cryotubes were taken out and rapidly thawed at 40±2°C for 2min. The seeds removed from the cryotubes and kept for germination in their optimized germination/regrowth medium. After 4 weeks of culture, the observation was made on the percentage of seed germination based on the emergence of new shoots and roots from the seed embryo. The experiments were repeated thrice with ten replicates per treatment. Statistical analysis was done by ANOVA ($P<0.05$) and means compared using Turkeys test (software Origin 7.0).

RESULTS

Effect of preculture solutions on cryotolerance

Of the various preculture solutions tested for protecting the seeds against cryopreservation, a preculture solution containing MS medium supplemented with 0.6M Sucrose and 2M Glycerol was found to be exclusively suitable for withstanding LN. Preculturing the seeds in this solution for 3h before dehydration played a vital role in successful recovery of seed germination after LN exposure. Seeds tested in other preculture solutions although could withstand dehydration at different moisture levels were unable to survive after exposure to LN.

The results mentioned below are for the seeds that were precultured in 0.6M Sucrose and 2M Glycerol.

Effect of dehydration time on moisture content and germination without exposing to liquid nitrogen (LN)

Seed germination of *M. spathulata* was significantly higher (70%) on the regrowth medium at the initial moisture level of 79.6% without dehydration i.e at 0h (Table 7.1). However, with subsequent increase in the dehydration time, the germination of seeds significantly decreased. When, the moisture content dropped down from initial 79.6% to 26.04% after 2h, a maximum of 63.3% seeds germinated on the regrowth medium. With the further increase in dehydration time, the moisture content and percentage of seed germination significantly decreased. A minimum of 36.6% seeds germinated on the regrowth medium after 6h of dehydration and finally the germination was completed inhibited at 8h of dehydration with 3.76% moisture content.

Similarly, for *M. wengeri,* significantly 73.3% seeds germinated on the regrowth medium without dehydration at an initial moisture level of 85.3% (Table 7.2). However, dehydrating the seeds for 2h resulted in the decrease in the moisture level to 20.77% and only 56.6% of seed germination was recorded in the regrowth medium. With further increase in the dehydration time, the percentage of moisture content and seed germination progressively decreased to minimum and only 10% germination was recorded at 6h dehydration with 6.5% moisture level. The seed germination was completely inhibited at 8h of dehydration.

Effect of storage in liquid nitrogen (LN)

Exposing the seeds of *M. spathulata* to LN without dehydration i.e 0h at 79.6% moisture content, the seeds failed to germinate in the regrowth medium. However, as the moisture level dropped down gradually at various time intervals (2h, 4h, 6h and 8h), the seeds were recorded to germinate significantly after 1h cryostorage in LN. Significantly, a highest of 40% seed germination was recorded on their regrowth medium with the decreased in the moisture content of seeds from 79.6% to 26.04% after 2h of dehydration (Table 7.1; Fig. 15 B). However, the germination of seeds was progressively decreased with further decrease in the moisture content at 4h, 6h and 8h, and was completely inhibited after 8h of dehydration.

Similarly, at an initial moisture level of 85.3%, the seeds of *M. wengeri* failed to germinate after cryostorage in LN for 1h. However, with gradual fall in the moisture level at 2h, 4h and 6h dehydrations, seeds were recorded to germinate after 1h cryoexposure to LN. With the decrease in the moisture level to 16.2% after 4h of dehydration, a maximum of 36.6% seeds were recorded to germinate on regrowth

Table 7.1: Percentage of seed germination of *M. spathulata* after cryopreservation through dehydration at different time intervals

Dehydration time (h)	Moisture content* (%)	Germination* (%)	
		Control	LN treatment
0.0	79.6±0.55	70±5.7[a]	0.0±0.0
2.0	26.04±1.5	63.3±3.3[a]	40.0±5.7[a]
4.0	19.01±0.88	46.6±3.3[b]	26.6±3.3[a]
6.0	9.3±0.73	36.6±3.3[b]	20.0±5.7[b]
8.0	3.76±0.1	0.0±0.0	0.0±0.0

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance, the germination of seed is highly significant.
Means followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 30 days of culture

Table 7.2: Percentage of seed germination of *M. wengeri* after cryopreservation through dehydration at different time intervals

Dehydration time (h)	Moisture content* (%)	Germination* (%)	
		Control	LN treatment
0.0	85.30±1.4	73.3±3.3[a]	0.0±0.0
2.0	20.77±0.86	56.6±3.3[a]	20.0±5.7[a]
4.0	16.2±0.42	50.0±0.0[b]	36.6±3.3[bc]
6.0	6.5±0.62	10.0±5.7[b]	6.6±3.3[b]
8.0	2.73±0.25	0.0±0.0	0.0±0.0

'*' indicates mean average values of three repeated experiments with standard error (±SE)
ANOVA at 5% level of significance, germination of seed is highly significant.
Means followed by the same letters (a, b, c) are not significantly different according to Turkey's test (p = 0.05)
Data recorded after 30 days of culture

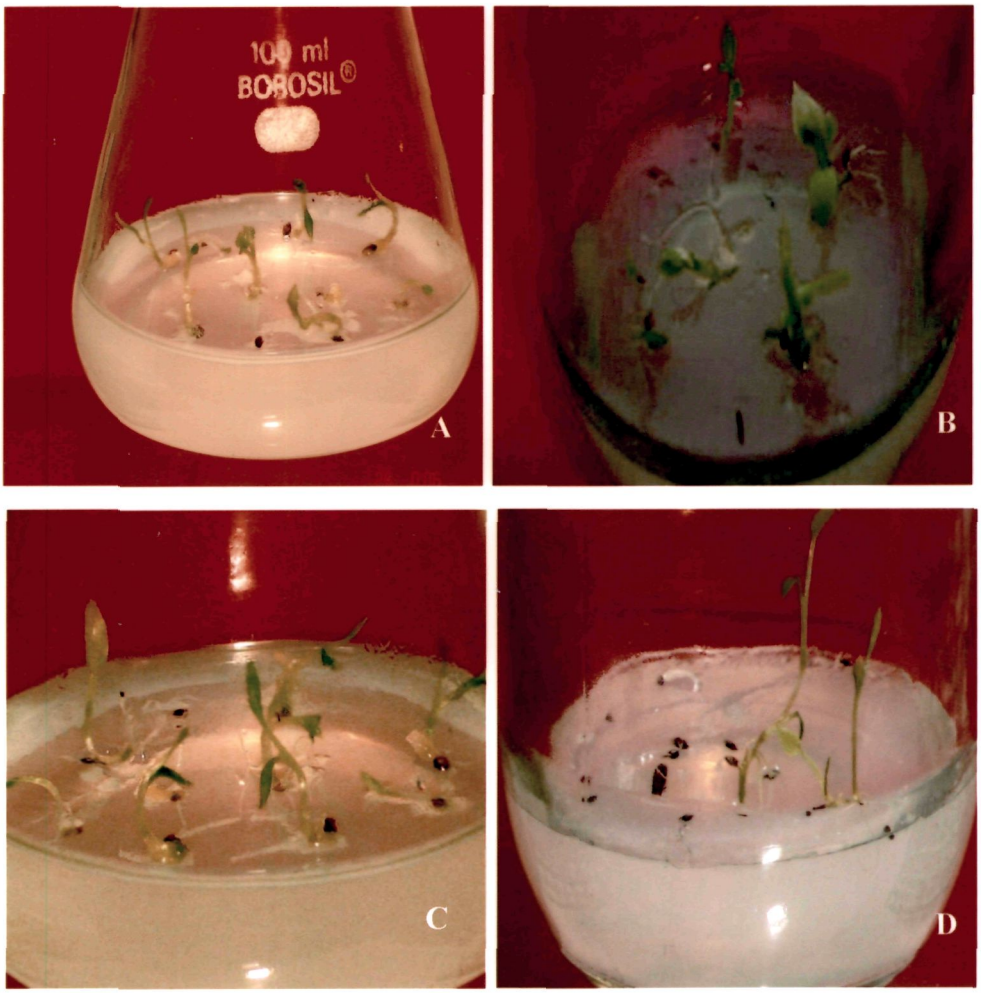


Fig. 15

medium which was significantly higher as compared to others treatments (Table 7.2; Fig. 15 D). At 6h dehydration, the moisture content decreased to a minimum of 6.5% and only 6.6% seeds were recorded to germinate in their regrowth medium. Seeds were not seen to germinate in the regrowth medium at 2.73% moisture content after 8h dehydration.

DISCUSSION

In the present study, the seeds of *M. spathulata* and *M. wengeri* were subjected to various treatments to investigate the optimal duration of dehydration. The seed moisture content is probably the most critical factor for developing successful cryopreservation protocol. As it relates to the composition of the seed, its storage behaviour and desiccation sensitivity imply precise levels of desiccation and tolerance to LN by species. Data suggest that the seeds of *M. spathulata* and *M. wengeri* tested can tolerate desiccation to a maximum of about 9.3% and 6.5% moisture content respectively and is critical beyond this level since the seeds cannot survive. Thus, from the present study, it is assumed that the seeds of both the species are sub-orthodox unlike desiccation tolerant orthodox seeds. As expected, seeds of none of the species tested were able to withstand immersion in LN at higher moisture level i.e. 79.6% and 85.3% moisture content for *M. spathulata M. wengeri* respectively.

Reports on the cryopreservation of seeds of zingibers are very limited, therefore preculture solutions that were reported to be suitable in other genus like citrus (Cho *et al.*, 2002a) were used for experimentations. Finally a preculture solution containing a mixture of 0.6M Sucrose and 2M Glycerol in combination was found to be exclusively suitable for the two species of *Mantisia*. The preculture

solution was found to be very crucial as the trials conducted without pretreating the seeds in this solution failed to survive LN exposure. Seeds of *M. spathulata* and *M. wengeri* can withstand dehydration and thus cryostored in LN with a maximum of 40% and 36.6% recovery in their regrowth medium. Similar, recovery to an extent of 75% has been reported in other endangered plants such as *Zizania texana* (Walters *et al.*, 2002). While freezing intact seeds of *Citrus aurantifolia,* the highest survival percentage (41.3%) was achieved after desiccation to 7.3% moisture content (Cho *et al.*, 2002 b). Pretreatment with 2M Glycerol and 0.6M Sucrose for 1h has been found to increase the survivability of seed embryos to 65% in *Citrus madurensis* after cryopreservation (Cho *et al.*, 2002 a). Sufficient number of capsules bearing numerous seeds has been obtained from the plants growing in the experimental garden. Thus, the protocol developed for cryopreserving the seeds of both the species would greatly help in long-term conservation of the seed diversity of these two critically endangered and endemic plants.

CHAPTER VIII: SUMMARY

Micropropagation and conservation of critically endangered *Mantisia spathulata* and *Mantisia wengeri,* two endemic plants of North-east India have been successfully achieved in the present study. An effective survey at different parts of the North-east India during the monsoon was conducted. There is no report on the survey of these plants for the past 20 years. Around 60 - 70 plants of *M. spathulata* and 40 - 50 plants of *M. wengeri* were found growing on the rocky hills along the road sides of two villages of Lunglei, viz. Lunglawn and Sethlun respectively at an altitude of around 1100 - 1300m above mean sea level. It was observed that the number of plants of the two species has severely declined in their natural habitats due to heavy landslides and rainfall. Therefore, experiments were carried out for multiplication of these two species using limited plant materials available from the nature. Out of the various explants used viz., juvenile leaves, roots etc., rhizomatous shoot buds explants were found to be only responsive for initiating aseptic cultures. This could probably be due to the presence of active cambial meristems in these tissues. After appropriate surface sterilization, the explants formed around 2 - 3 aseptic shoot buds within 3 - 4 weeks from the nodal portion of rhizomatous bud in MS medium incorporated with a combination of 8.8μM BA and 2.7μM NAA (*M. spathulata*) and 4.4μM BA and 2.7μM NAA (*M. wengeri*). The initial primary cultures thus obtained for both the species were further multiplied in their respective initiation medium to obtain

sufficient explants. Finally, experiments were carried out to study the effect of different physio-chemical factors viz., growth regulators, photoperiod, light, temperature etc. required for optimizing the shoot multiplication, growth and development of the *in vitro* plantlets. In the present study, it was found that explants of *M. spathulata,* regenerated with a highest of 6.1±0.55 BFC in MS medium supplemented with 10μM BA and 2.5μM of NAA. Similarly, 7.82±0.73 BFC was achieved for *M. wengeri* in MS medium containing 5μM BA and 2.5μM of NAA. The parameters used for measuring the growth and development of the *in vitro* plantlets of both the species were also found to be optimum at these treatments. The efficacy of shoot multiplication, growth and development of *in vitro* plantlets in medium containing other growth regulators showed varying responses. Incubating the cultures at a temperature of 24±2°C under 12h daily illuminations with white fluorescent light of 40.5μmoles $m^{-2}s^{-1}$ was found to optimize the shoot multiplication, growth and development of the *in vitro* plantlets of both the species. More than 5000 plantlets of *M. spathulata* and *M. wengeri* were regenerated *in vitro* in multiplication medium under optimum growth conditions.

The transfer of plantlets from the culture vessels to the glasshouse conditions requires a careful and stepwise procedure. Successful transplantation also depends on suitable size of the plantlets and their state of growth *in vitro*. Healthy plantlets showing vigorous growth in the culture vessels were transferred to the pots. Out of the different substrata used for hardening and acclimatization of both *M. spathulata* and *M. wengeri* in this investigation, it was found that equal ratios (1:1) of soil and compost to be most suitable for higher survivability of transferred plants. Around 90.7% plantlets of *M. spathulata* and 84% of *M. wengeri* got acclimatized in this

compost. Exposing the plantlets gradually to a relatively lower humidity, higher temperature and higher light intensity also maximized the survivability of the *in vitro* plantlets during acclimatization.

In the present study, the plantlets were found to be morphologically similar, however tissue culture-raised plantlets are very often associated with somaclonal variations as reported in many cases. Any cryptic variants arising out of cultures might not help in the conservation of rare and endangered plants as these variants may become lethal for their survivability later. Therefore, investigation was carried out to assess the genetic fidelity of *in vitro*-raised hardened plantlets of *M. spathulata* and *M. wengeri* before their transfer to the field conditions. RAPD markers were used to evaluate the genetic stability of *in vitro*-raised hardened plantlets as it can detect single base change in genomic DNA. Similarity coefficient among the regenerated plants ranged between 0.85 - 0.98 for *M. spathulata* and 0.83 - 0.98 for *M. wengeri*. A maximum of 88% and 90% genetic similarity were obtained between *in vitro* raised hardened plantlets and mother stock of *M. spathulata* and *M. wengeri* respectively. There are many reports of 80 - 90% similarities between *in vitro* and mother plants which are considered to be normal. Thus, in the present study, the plants of both the species showing these similarities could be categorized as normal plants and equivalent to mother stock with regard to their genetic make up.

The success of any conservation measures depends upon the rate at which the plants survive in the natural habitat after reintroduction. As reported in other species maximum loss occurs during the phase when plants get acclimatized in the natural habitats due to several biotic as well as abiotic factors. A trial on the reintroduction of the plant species was carried out by transferring the hardened plantlets in the natural

habitats for their recovery. Around 52% and 45% of *M. spathulata* and *M. wengeri* plants survived respectively under natural climatic conditions of Lunglei, Mizoram after reintroduction. As a measure of *ex situ* conservation the hardened plantlets were also introduced in the Experimental Garden of the Plant Biotechnology laboratory, Department of Botany, North Eastern Hill University, Shillong. More than 90% plantlets of both the species introduced in the Experimental Garden survived without noticeable morphological variations. It is assumed from the study that the most of the ecological factors like precipitation, temperature, humidity, light intensity etc. prevailing in the natural habitats must be similar in the reintroduced area of Shillong, which allowed the higher success rate of reintroduction of the two species in Shillong.

In the present study, it was observed that new young seedlings were not seen to grow in and around the areas where the seeds from the capsules dispersed. Hence, it could be assumed that the seeds fail to germinate in nature either due to abiotic or biotic factors. The low percentage of seed germination of around 19.7% and 24.2% under *in vivo* condition for *M. spathulata* and *M. wengeri* respectively in the soil in the present study proves it. For the conservation of these species it was also desirable to propagate plants through seeds so as to maintain the heterogeneity which would lead to the production of diversity in the germplasm of seedlings. Therefore, the seeds collected from the nature were germinated under *in vitro* conditions in culture medium supplemented with growth regulators. Germination of the seeds was observed to be significantly enhanced to 90% and 96.7% for *M. spathulata* and *M. wengeri* in MS medium supplemented with 4.3μM and 7.2μM of GA_3 respectively. To propensate the diversity of these rare species, the seed derived plantlets of *M.*

spathulata and *M. wengeri* obtained under *in vitro* conditions were finally transferred in the experimental garden.

Long-term conservation of *M. spathulata* and *M. wengeri* has been successfully achieved through cryopreservation of the seeds. Cryopreservation provides an important practical approach for germplasm conservation as compared to *in vitro* cultures. Although different tissues such as shoot tips, protocorm-like bodies (PLBs), meristems etc. have been used, seeds of many species were found to be most suitable due to their inherent low moisture content and therefore, been easily cryopreserved under LN in most of the cases. Seeds of *Mantisia* species after pretreatment with higher osmoticum were dehydrated under sterile air of clean bench. With the fall of moisture level to 26% in the seeds after 2h of dehydration, around 40% seeds of *M. spathulata* were recovered in the regrowth medium after cryopreservation. Similarly, when the seeds of *M. wengeri* were dehydrated to 16.2% moisture level after 4h, around 36% recovery was possible in regrowth medium after cryostoring in LN for 1h.

The conservation and recovery of these two rare and endemic zingibers has opened up the possibilities of conducting various significant studies in future. The rhizome of these plant species has been used as a remedy for bone fracture and gastro-intestinal ailments in the past by local people. Therefore, serious efforts must be paid to explore the phyto-chemical constituents of these rare zingibers.

Studies on the developmental biology of the species of *Mantisia* are very essential due to their unique flowering characteristics. Flowers appear before the onset of vegetative shoots during monsoon. The vegetative cycle is only for six months and the rhizomes remain dormant in the later part of the year. From our preliminary

studies on the plants introduced in the experimental garden, it was observed that the dormant rhizomes are destined to produce a single inflorescence for the successive year after pre-monsoonal showers. However, not all the rhizomes are able to form floral inflorescences and instead form vegetative shoots.

The pollination studies for these plants are very urgent as most of the flowers produce capsules bearing seeds. To our observations insect pollinators, mostly bees, are observed to cross the flowers. Therefore, detailed study on the pollination of these ornamental flowers should be carried out to understand interesting phenomenon of plant-animal interaction for these rare zingibers. It was also observed that before getting detached from the floral stock the seed-bearing capsules burst and disperse the seeds in the soil which are eaten by ants. New seedlings were not found to emerge in the soil during the same or successive year. Therefore, extensive studies must be carried out for finding the reasons for the non-germinability of the seeds in the soil.

As the species adapted rapidly under the climatic conditions of Shillong, further reintroduction of these species in other parts should be attempted using Niche Ecological Modelling Tools.

CHAPTER IX: REFERENCES

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High frequency plantlet regeneration from rhizomatous buds in *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer and analysis of genetic uniformity using RAPD markers

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A protocol has been devised for enhanced *in vitro* regeneration of critically endangered *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer. Highest Bud Forming Capacity (BFC) of 6.10±0.55 with an average of 19.93±3.19 roots was obtained for *M. spathulata* within 5-6 weeks in Murashige and Skoogs (MS) medium supplemented with a combination of 10.0μ*M* of N^6-benzyladenine (BA) and 2.5μ*M* of α-naphtalene acetic acid (NAA). For *M. wengeri*, BFC of 7.82±0.73 and 20.86±1.65 roots was achieved in MS media supplemented with a combination of 5.0μ*M* BA and 2.5μ*M* of NAA RAPD markers were used to evaluate the genetic stability of *in vitro* raised hardened plantlets. Similarity coefficient among the regenerated plants ranged between 0.85-0.98 for *M. spathulata* and 0.83-0.98 for *M. wengeri*. Maximum of 88 and 90% genetic similarity were obtained between *in vitro* raised hardened plantlets and mother stock of *M. spathulata* and *M. wengeri*, respectively through RAPD analysis. The hardened plantlets after RAPD analysis on being transferred to soil of experimental garden showed no marked phenotypic variations in vegetative or floral characteristics.

Keywords: *Mantisia spathulata*, *Mantisia wengeri*, Plant regeneration, RAPD analysis

Mantisia spathulata and *M. wengeri* are two critically endangered zingibers native to Mizoram, a North-eastern state of India. This area of India falls under the most poorly explored regions in Asia for plant diversity of all types[1] including many unique rare zingibers. Commonly known as 'dancing girl', *Mantisia* species are perennial herbs and therefore, floral spikes bearing numerous splendid flowers of *M. spathulata* (pale violet with yellow lip) and *M. wengeri* (yellow colour) appear before the onset of vegetative shoots during early monsoon. Due to natural calamities and human intervention, *M. spathulata* and *M. wengeri* have become critically endangered in the natural habitat [2] and are listed in the Red Data Sheet of rare and endangered Indian plants (www.envfor.nic.in/bsi/research.html). The rarity of *M. wengeri* has reached a critical level and has been included in the national priority list for its recovery by Department of Biotechnology, New Delhi, India [3]

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For mass propagation and rapid recovery of rare and endangered zingibers, tissue culture techniques have been effectively used world wide [4-8]. However, the regenerants arising out of *in vitro* cultures are susceptible to genetic changes due to culture stress and mode of regeneration[9-11]. To minimize the risk of any cryptic variations arising out of *in vitro* cultures, it is crucial to assess the genetic uniformity of the *in vitro* plantlets with mother stock before their transfer to the field conditions. DNA markers offer a more attractive means for examining genetic similarity/dissimilarity since these markers are not developmentally regulated. Usefulness of Random Amplified Polymorphic DNA (RAPD) in detection of variation has been amply demonstrated in large array of *in vitro* plants by many workers[11-14]. Present report deals with the *in vitro* regeneration and RAPD analysis for finding genetic similarity of the *in vitro* regenerants of *M. spathulata* and *M. wengeri* with their respective mother stock.

Materials and Methods

Initiation of aseptic primary cultures—For initiating aseptic cultures, juvenile rhizomatous buds of *M. spathulata* and *M. wengeri* (collected from their natural habitats and maintained in the glass house of

the Department) were washed in running tap water for 1h along with few drops of detergent (Tween-20, High Media, India). The buds were surface disinfected using 0.2% cetramide followed by surface sterilization with 0.1% $HgCl_2$ for 5min. Rhizomatous bud explants measuring 2.0 × 2.0cm were dissected out and cultured in tubes containing 20ml of Murashige and Skoogs (MS)[15] medium supplemented with 3% sucrose and BA (0.0, 4.4, 8.8, 13.2, 17.6, 22.2μ*M*) in combination with NAA (0.0, 2.7, 8.1, 16.2, 26.8μ*M*). The cultures were incubated at 25°±2°C under 12h photoperiod with a photosynthetic photon flux density (PPFD) of 40.5μmole $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. Same cultural practices were followed for both the plants in all the experiments conducted. The miniature rhizomes from the *in vitro* aseptic shoots (0.5 - 1.5cm) were dissected out and sub cultured in their respective initiation medium so as to obtain sufficient cultures for standardizing the protocol for high frequency *in vitro* plantlet regeneration.

In vitro regeneration of plantlets—Rhizomatous bud explants (~ 2.5 × 2.5mm in size) from 4 weeks old *in vitro* - raised primary cultures were inoculated in MS medium supplemented with different concentrations of BA (2.5, 5.0, 10, 20 μ*M*) and NAA (2.5, 5.0, 10, 20μ*M*) in combination so as to optimize the best concentration for multiplication of shoot buds in the secondary cultures. Regeneration of shoot buds from each explant was calculated as the bud forming capacity using the formula, BFC = (average no of buds per explant) × (% of explants forming buds) ÷ 100 (Ref.8). Observation on percentage response, BFC and root number were made after 45 days of culture.

Statistical analysis—Ten replicates were maintained for each treatment and the experiment repeated thrice. Statistical analysis was done by Analysis of Variance (ANOVA) at 5% significant level and means compared using Tukeys test (PC version Origin 7.0. NORTHAMPTON, MA, USA).

Hardening and field transfer of plantlets—About 2 months-old *in vitro* raised plantlets, measuring 2.5-3.0cm in size were removed from culture vessels and washed with water to remove the agar containing medium. The plantlets were then potted to paper cups containing mixtures of soil and compost (1:1). The cups were covered with perforated polybags and sprinkled with water alternately at two days interval for the initial two weeks and acclimatized at 30°±2 °C and 70±5% RH under glass-house conditions. The established hardened plantlets were removed from the paper cups and transferred to the experimental garden after one year.

Random amplified polymorphic DNA (RAPD) analysis of the hardened plantlets—For RAPD analysis, five 6-month old hardened plantlets were randomly selected and compared for genetic similarity with mother stock of both the species. DNA was extracted with DNeasy Plant Mini Kit (QUAGEN) using 100mg of fresh leaves for each sample and dissolved in 100μl of buffer AE which yielded total DNA of ~50ng/μl DNA (1μl) was loaded in 1% (w/v) agarose gel and electrophoresed under 60V constant power supply for 3h and the amplification products were visualized in Geldoc (UVP biodoc-IT) system. In a pre-screen with 30 primers based on amplification of mother plant, 5 arbitrary decamer primers (Operon Technologies, USA) produced easily scorable distinct amplification profiles that were reproducible and hence selected for polymerase chain reaction (PCR). PCR was performed in a reaction volume of 25μl containing ~ 50ng template DNA, 1X Taq buffer A containing 1.5m*M* $MgCl_2$, 60μ*M* of each dNTP's, 0.05 units *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.) and 0.6ng primers (Operon Technologies, USA). Amplification was performed in a programmed thermal cycler (2700) supplied by Applied Biosystems (USA). After initial denaturation at 92°C for 3.30min., PCR was operated for 45 cycles consisting of a denaturing step (1min.), 35°C primer annealing step (1.30min.) and 72°C amplification (2min) step, at the end of the run final amplification period of 7min (72°C) was appended. Amplification products were separated in 1.5% agarose gels in 1X TBE buffers stained with ethidium bromide and photographed with UVP biodoc-IT system. The electrophoretogram was scored for the presence of a band (1) or its absence (0). The data were analysed using SIMQUAL (similarity for qualitative data) routine to generate Jaccard's similarity coefficient. These similarity coefficients were used to generate dendrograms using Unweighted Pair Group Methods with Arithmetic averages (UPGMA) employing the Sequential Agglomerative Hierarchic Non-overlapping (SAHN) programmes from NTSys PC version 2.02 k software. Similarity matrix was compared with dendrogram using MxComp and correlation coefficient of the association was obtained to determine the significance level.

Results

Initiation of aseptic primary cultures—Around 20 and 40% of rhizomatous bud explants of

M. spathulata and *M. wengeri* produced contamination-free rooted shoots, respectively. Axillary shoot buds (2-3) sprouted within 3-4 weeks (Fig. 1A, Fig. 2A) from the nodal portion of rhizomatous bud explants on initiation medium [MS supplemented with 8.8μ*M* BA+2.7μ*M* NAA for *M. spathulata* and BA (4.4μ*M*) + NAA (2.7μ*M*) for *M. wengeri*]. However, the explants of both the species remained non responsive even after 8 weeks of culture in MS medium incorporated with other concentrations of BA and NAA in combinations as well as in the control.

In vitro regeneration of plantlets—Bud regeneration potentiality was maximized by using the secondary explants obtained from primary cultures. Highest BFC of 6.10±0.55 with an average of 19.93±3.19 roots was obtained within 5-6 weeks for *M. spathulata,* in MS medium supplemented with BA (10.0μ*M*) and NAA (2.5μ*M*) (Fig. 1B, Table 1). A combination of BA at higher concentrations of NAA reduced the bud forming capacity and formed clumped roots. Similarly, BFC of 7.82±0.73 with an average of 20.86±1.65 roots was achieved within 5-6 weeks for *M. wengeri* in MS medium containing BA (5.0μ*M*) and NAA (2.5μ*M*) (Fig. 2B, Table 1). Although the BFC of *M. wengeri* got significantly reduced at higher concentrations of BA and NAA in combinations, the roots formed were normal. However, the roots of *M. spathulata* got clumped into globular mass at higher concentrations of BA and NAA. In both the species, the explants kept in MS control (devoid of any growth regulators) although showed more than 90% response, the BFC and root regeneration was however significantly less in comparison to medium supplemented with growth regulators.

Hardening and field transfer of plantlets—More than 90% plantlets of both the species got hardened and acclimatized within 8 weeks for both the species (Fig. 1C, 2C). So far all the plantlets transferred to field, established without any loss of plantlets and maintained for past two years.

RAPD analysis of hardened plantlets—Size of bands produced by the primers ranged from 500 bp with primers OPA 2, OPA 3, OPC2, OPC 8 and OPC 15 to 3250 bp with primer OPA 2 for *M. spathulata* (Fig. 3). On the other hand, the size of bands produced by the same primers ranged from ~ 800 bp

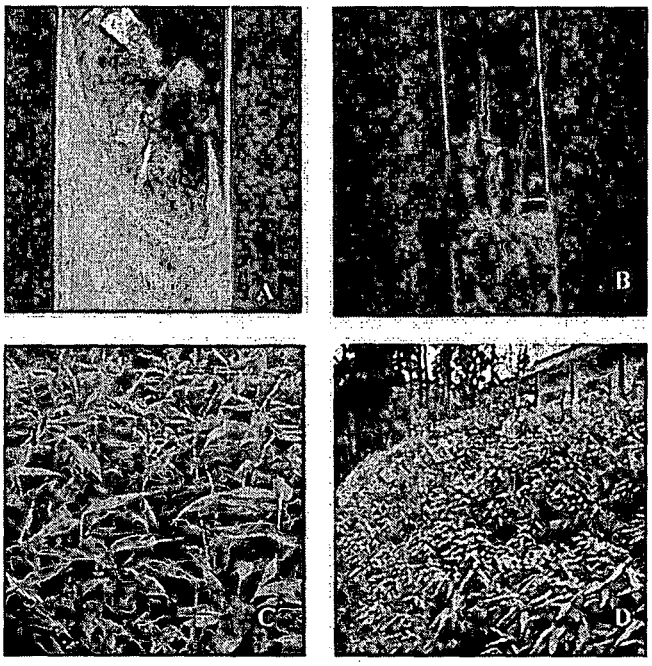

Fig. 1—(A)–Initiation of primary cultures of *M. spathulata* in MS medium containing BAP (8.8μ*M*) + NAA (2.7μ*M*) within 3 - 4 weeks; (B) Regeneration of *M. spathulata* in MS medium supplemented with BAP (10.0μ*M*) and NAA (2.5μ*M*) within 5 - 6 weeks; (C) Hardened plantlets of *M. spathulata* after 6 months in glass house; and (D) Re-established plantlets of *M. spathulata* in the experimental garden after two years

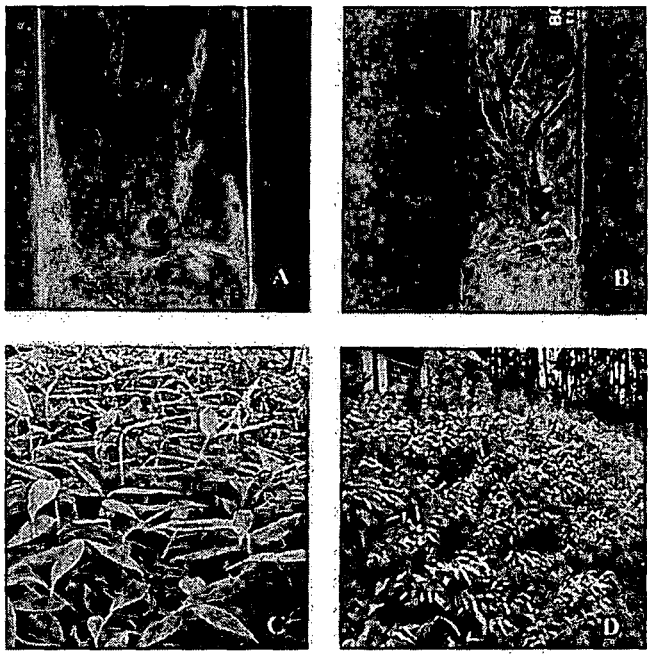

Fig. 2—(A)–Initiation of primary cultures of *M. wengeri* in MS medium containing BAP (4.4μ*M*) + NAA (2.7μ*M*) within 3 - 4 weeks; (B) Regeneration of *M. wengeri* in MS medium supplemented with BAP (5.0μ*M*) and of NAA (2.5μ*M*) within 5 - 6 weeks; (C) Hardened plantlets of *M. wengeri* after 6 months in glass house; and (D) Re-established plantlets of *M. wengeri* in the experimental garden after two years

with primers OPC 15 to ~ 3000 bp with primer OPA 2, OPA 3, OPC 2, OPC 8 and OPC 15 for *M. wengeri* (Fig. 4). From the analysis of the data the test for association using MxComp yielded the matrix correlation coefficient (r) as 0.917 and 0.778 for *M. spathulata* and *M. wengeri* respectively which was highly significant. From SIMQUAL generated matrix data it was observed that similarity coefficient ranged between 0.85-0.98 among regenerated plants of *M. spathulata* and 0.83-0.98 among regenerated plants of *M. wengeri* (Table 2). From the matrix generated dendrogram it was ampy clear that all the *in vitro* raised plants of both *M. spathulata* and *M. wengeri* had shown distant similarities with mother plant stock (Figs. 5, 6).

Discussion

In vitro induction and multiplication of shoots represents the first step in micropropagation[16]. Raising aseptic cultures from underground rhizomes in zingibers happen to be a challenging task due to excessive contamination of explants under *in vitro* conditions as reported in *Zingiber officinale* Rosc[17]. Once aseptic shoots buds are induced the successive multiplication rate gets maximized under *in vitro* conditions in most of zingibers such as *Zingiber officinale*, Rosc and *Curcuma longa*[18]. Efficiency of rhizomatous buds for high shoot bud regeneration has been reported in large number of other zingibers such

Table 2—Similarity coefficient among mother plant and *in vitro* raised hardened plantlets of *M. spathulata* and *M. wengeri* based on RAPD markers

	In vitro plantlets					
	1*	2	3	4	5	6
(*M. spathulata*)						
1*	1.00					
2	0.91	1.00				
3	0.85	0.89	1.00			
4	0.89	0.98	0.91	1.00		
5	0.87	0.96	0.93	0.98	1.00	
6	0.87	0.96	0.93	0.98	0.96	1.00
(*M. wengeri*)						
1*	1.00					
2	0.93	1.00				
3	0.98	0.91	1.00			
4	1.00	0.93	0.98	1.00		
5	0.90	0.83	0.88	0.90	1.00	
6	0.95	0.88	0.93	0.95	0.95	1.00

1*- Mother plant

Table 1—Effect of BA and NAA in MS medium on the regeneration of *M. spathulata* and *M. wengeri* [Values are mean ± SE of three experiments with ten replicates/experiment]

BA+NAA (μ*M*)	Response of *M. spathulata* (%)	BFC	Root no.	Response of *M. wengeri* (%)	BFC	Root no.
Control	93.3±6.6^{a}	1.42±0.1^{a}	3.0±0.4^{a}	93.3±6.6^{a}	2.09±0.44^{a}	4.3±1.05^{a}
2.5+2.5	93.3±6.6^{a}	3.16±0.4^{a}	7.8±0.75^{a}	73.3±6.6^{a}	2.04±0.37^{a}	13.8±1.82^{bc}
5.0+2.5	93.3±6.6^{a}	4.25±0.6^{b}	11.0±0.90^{b}	93.3±6.6^{a}	7.82±0.73^{bc}	20.86±1.65^{bc}
10.0+2.5	93.3±6.6^{a}	6.10±0.5^{bc}	19.93±3.19^{bc}	93.3±6.6^{a}	5.73±0.58^{b}	13.46±0.89^{bc}
20.0+2.5	93.3±6.6^{a}	2.60±0.5^{a}	18.40±0.87^{bc}	93.3±6.6^{a}	3.36±0.50^{a}	8.03±1.33^{a}
2.5+5.0	66.6±6.6^{a}	1.33±0.3^{a}	11.73±1.09^{b}	60.0±11.5^{b}	1.32±0.56^{a}	6.86±1.43^{a}
5.0+5.0	73.3±6.6^{a}	2.25±0.4^{a}	15.83±1.85^{bc}	73.3±6.6^{a}	2.45±0.41^{a}	7.93±0.75^{a}
10.0+5.0	60.0±0.0^{a}	2.28±0.3^{a}	17.56±2.01^{bc}	86.6±6.6^{a}	2.46±0.60^{a}	10.9±0.86^{a}
20.0+5.0	53.3±6.6^{b}	1.32±0.1^{a}	12.9±1.86^{bc}	93.3±6.6^{a}	3.93±0.83^{a}	4.73±0.73^{a}
2.5+10.0	60.0±0.0^{b}	0.85±0.1^{a}	6.1±1.7^{a}	66.6±6.6^{a}	1.25±0.43^{a}	18.93±2.46^{bc}
5.0+10.0	40.0±0.0^{b}	1.32±0.1^{a}	CM	80.0±11.5^{a}	2.02±0.63^{a}	14.8±2.45^{bc}
10.0+10.0	40.0±0.0^{b}	0.32±0.0^{a}	CM	80.0±11.5^{a}	2.30±0.64^{a}	12.0±1.5^{a}
20.0+10.0	40.0±0.0^{b}	0.25±0.0^{a}	CM	73.3±13.3^{a}	1.49±0.66^{a}	7.86±0.78^{a}
2.5+20.0	46.6±6.6^{b}	0.24±0.0^{a}	CM	20.0±0.0^{b}	0.08±0.08^{a}	2.73±1.39^{a}
5.0+20.0	-	-	-	20.0±0.0^{b}	0.1±0.07^{a}	3.16±2.32^{a}
10.0+20.0	-	-	-	26.6±6.6^{b}	0.5±0.46^{a}	3.6±1.97^{a}
20.0+20.0	-	-	-	26.6±6.6^{b}	0.3±0.26^{a}	0.66±0.16^{a}

Values followed by the same letter are not significantly different according to ANOVA ($P \leq 0.05$) and Tukey's test; - no response; CM clump

as *Zingiber officinale*[17], *Curcuma longa*[18], *Alipinia galanga*[5] etc. An optimum concentration of BA in combination with NAA has been found to be very effective in high shoot bud proliferation with simultaneous root formation in rare species like *Kaempferia galanga*[6] and *Zingiber petiolatum*[7]. Use of BA in combination with NAA has also been found to be effective in the enhancement of shoot buds and root formation in our investigation with the two species.

Genetic variation from *in vitro* cultures has been reported in some species. Variations to an extent of 26% have been reported in micropropagated plants of *Populus deltoides*[19]. Similarly, there are reports of

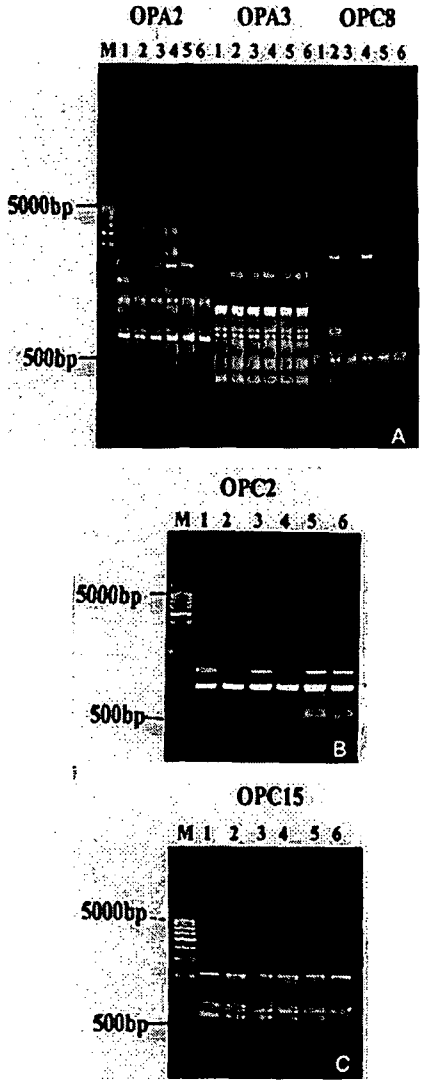

Fig. 3—RAPD profiles of *M. spathulata* using primers OPA2, OPA3, OPC8 OPC2 and OPC15, M-500 bp markers, Lane 1–mother plant, Lane 2, 3,4,5,6 – *in vitro* regenerated hardened plantlets

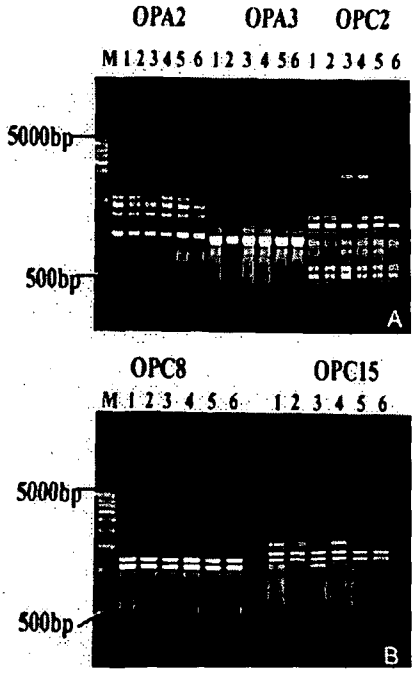

Fig 4—RAPD profiles of *M. wengeri* using primers OPA2, OPA3, OPC2, OPC8and OPC15, M- 500 bp markers, Lane 1–mother plant, Lane 2, 3,4,5,6–*in vitro* regenerated hardened plantlets

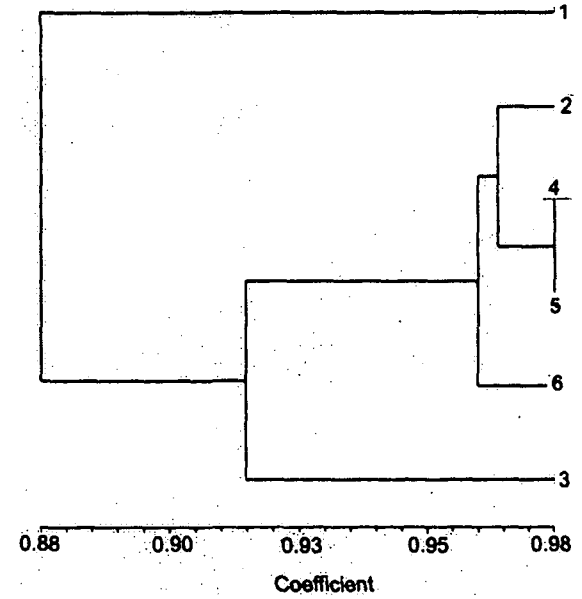

Fig. 5—Dendrogram showing the relationship of *in vitro* regenerated hardened plantlets of *M. spathulata* with their mother stock

genetic similarity coefficients in the range of 84-97% in field transferred plants of *Angelica acutiloba*[20] and 86-96% in shoot bud regenerated micropropagated plants of *Robinia pseudoacacia*[11]. The present estimations of about 88 and 90% similarity for *M. spathulata* and *M. wenger*, respectively with their mother plant are near to the acceptable level. By this

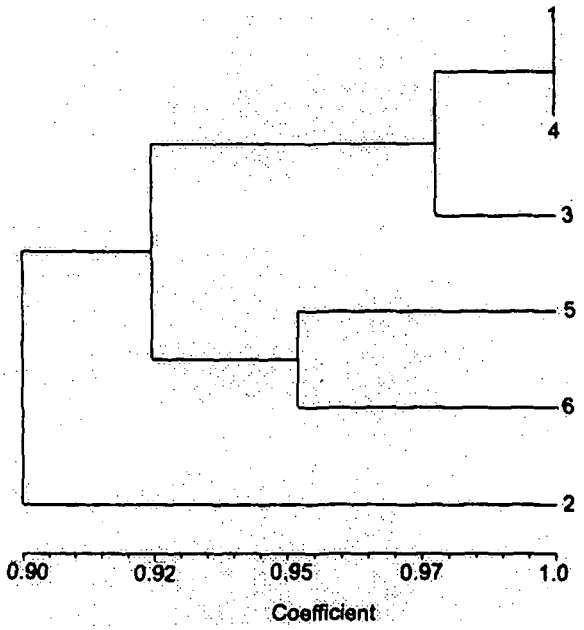

Fig. 6—Dendrogram showing the relationship of *in vitro* regenerated hardened plantlets of *M. wengeri* with their mother stock

parameter the regenerated plants produced presently for both *M. spathulata* and *M. wengeri* can be categorized as normal plants and equivalent with mother stock with regard to genetic make up.

Genetic variations under *in situ* conditions in most of zingibers including *Mantisia* are sluggish due to the clonal propagation through rhizomes[18] and lack of seed propagation[21, 22]. In this regard, minor genetic variations arising out of *in vitro* cultures without marked phenotypic alterations are expected to be beneficial for faster adaptability under varied climatic conditions and prolonged sustainability under biotic and abiotic stresses. Around 500 hardened plantlets of both the species after being transferred to the soil of experimental gardens got acclimatized under the identical climatic conditions of Lunglei, Mizoram which are prevalent in Shillong, Meghalaya of North-eastern India, where the experiments were conducted (Fig. 1D, 2D). Around 80% of the plantlets flowered after 2 years of field transfer without any marked phenotypic variations in floral or vegetative characteristics. Therefore, our novel strategy for large scale *ex situ* conservation of the two endemic plant species in the experimental garden is of paramount interest as it would lead to sustainability of the germplasm for many years to come.

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Rapid *in vitro* Clonal Propagation of *Mantisia spathulata* Schult, A Rare and Endemic Plant of Northeastern India for Recovery

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Abstract: *Mantisia spathulata* (Zingiberaceae) is a rare and endangered, endemic plant of North-East India. The plant has been rediscovered from Mizoram, India after two decades. Successful recovery of the plant has been achieved through an efficient true-to-type micropropagation protocol. Various concentrations of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) were tried for *in vitro* plantlet production from rhizomatous shoot explants. Optimum shoot and root multiplication was obtained in 8 weeks using rhizomatous shoot explants cultured on Murashige and Skoog medium supplemented with 11.1 μM BAP and 2.7 μM NAA. The *in vitro* plantlets were successfully weaned and transferred to soil with about 90-93% survival rate. So far, more than 1000 plants have been produced successfully and reintroduced into nature for the recovery of this species.

Key words: Conservation, *ex situ*, *in vitro*, *Mantisia spathulata*, rhizome, Zingiberaceae

INTRODUCTION

The genus *Mantisia* (Zingiberaceae) comprising only four species viz. *M. spathulata* Schult, *M. radicalis* Dam and Dam, *M. wardii* Smith and *M. wengeri* Fischer is endemic to the Hilly areas of the Northeastern parts of India and Myanmar (Dam *et al*., 1997). Due to excessive deforestation, human settlements, burning of forest and shifting cultivation practices, *M. spathulata* has become very rare in the last several years. The rarity of this plant has reached to such a level that only countable few representatives are available. The plant is listed in the Red Data sheet of Indian plants as being rare and endangered species by Botanical Survey of India (www.envfor.nic.in/bsi/research.html) and is found only in the rocky hills of Lunglei, Mizoram, at 1200-1500 m above the sea level (Dam *et al*., 1997). Therefore, immediate steps are needed to propagate and conserve this plant species, which is possible through micropropagation from limited plant material available for experimentation. Recent study has also suggested that the Zingibers, *Globba* and *Mantisia* are phylogenetically related and for understanding this complex group of plants increased field collections of *Globba* and its relatives are critical as more new species will certainly be discovered (Williams *et al*., 2004). Some rare and endangered plants of Northeast India such as *Coptis teeta* (Tandon and Rathore, 1992), *Nepenthes khasiana* (Rathore *et al*., 1991) and *Lilium mackliniae* Sealy (Mao *et al*., 2002) have been conserved successfully through micropropagation in the last several years. A survey of literature shows that the micropropagation has been achieved in a number of plants belonging to the family Zingiberaceae (Hosoki and Sagawa, 1977; Borthakur *et al*., 1998; Shirin *et al*., 2000; Prathanturarug *et al*., 2004). Most of the Zingiberaceae plants are perennial and flower for a short period in their natural habitats during monsoon. The vegetative parts of rare Zingiberous plants are similar and therefore proper identification and collection can be done only during their blooming period. The floral shoot of *Mantisia spathulata* emerges before the onset of the vegetative shoots and results in the formation of floral buds. Flowers are highly ornamental with white-purple petals, yellow-lips and resemble a dancing girl that lasts for only 2-3 days producing immature reproductive capsules. The rhizome has a dormancy period and sprouts during the monsoon and therefore vegetative propagation of the plant is limited in natural conditions. Present report deals with the resurvey and collection of *Mantisia spathulata* Schult from the natural habitats after two decades and its recovery through *in vitro* clonal propagation.

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MATERIALS AND METHODS

Collection and initiation of aseptic cultures: A resurvey for finding the natural habitats of *M. spathulata* was conducted after a gap of two decades during the month of May at Lunglei, Mizoram, India. Two plants were collected from the natural habitat and maintained at the glass-house of North Eastern Hill University, Shillong, India. Newly sprouted rhizomatous shoots (20-30 mm) were thoroughly washed in running tap water for 60 min along with few drops of surface-disinfectant, Tween-20 (Merck, India). Finally, these were surface-sterilized with 0.2% cetramide (Hi-media, India) for 8 min followed by 0.1% mercuric chloride solution for 5-6 min. The explants were rinsed several times with sterile pure water to remove all the traces of mercuric chloride. Under aseptic conditions the leaf sheathes were removed from the shoots and an explant of size 5×10 mm was dissected by a transverse cut at the nodal point of rhizome. A single explant was inoculated in 20×150 mm test tube containing 15 mL of sterilized MS (Murashige and Skoog, 1962) initiation medium supplemented with BAP (2.2-22.2 μM) and NAA (0.5 μM) containing 0.8% (w/v) extra pure agar (Hi-media, India). MS medium with 3% (w/v) sucrose without growth regulator was used as a control. The pH of the medium was adjusted to 5.8, prior to autoclaving for 15 min at 1.06 kg cm^{-2} (121°C). The cultures were incubated at a temperature of 25±2°C under 12 h daily illuminations with white fluorescent light of 50 $\mu moles^{-2}$ sec^{-1} intensity. The *in vitro* shoots obtained in the induction medium were used for further multiplication.

Multiplication of *in vitro* shoot and root: The meristematic rhizomatous shoots (10-15 mm) obtained in the induction medium were cut into 2×5 mm sizes and inoculated in MS medium supplemented with a range of growth regulators, viz., BAP (0.0, 2.2, 11.1, 22.2, 44.4 μM) and NAA (0.0, 2.7, 13.4, 26.9, 53.7 μM) both singly and in combinations. Culture conditions and media used were similar to the induction medium. Observations were made on the shoot and root number per explant after 7-8 weeks of culture.

Statistical calculations: All experiments were repeated thrice with ten replicates per treatment. The data were analyzed using one-way analysis of variance (ANOVA) and comparison between the mean values of treatments were made by the Least Significant Difference (LSD) test. Multiplication ratio of the plantlets was calculated following the method of (Nadgauda *et al.*, 1978).

Weaning and transplantation: Eight weeks old plantlets were removed from the culture bottles, washed and transferred to plastic pots containing a mixture of soil, sand and humus in the ratio of 1: 0.5: 0.5. The plantlets were acclimatized inside the glass-house at 25±2°C at 80% RH initially for a week and then at 30±2°C under 60% RH for the next week. Established plants were then grown under natural conditions after two weeks. The weaned plantlets were then reintroduced in the natural habitat for recovery.

RESULTS AND DISCUSSION

Collection and establishment of aseptic cultures: After a gap of three years, plants of *Mantisia spathulata* flowered in the natural habitat (Fig. 1A and B) of Lunglawn at an altitude of 1183 m, longitude 92° 45' 30, Latitude 23° 52'30, Lunglei District, Mizoram (Fig. 1C). Thus, *Mantisia spathulata* could be successfully identified among other Zingiberous plants and collected from their natural habitats. A single flower remained for 2-3 days with the formation of reproductive capsules within a week that burst off before maturity. The dispersed immature seed does not germinate in the natural habitat due to infestation and fungal contamination. The plant was found growing in the rocky hills on weathered clayey soil containing slit that undergoes frequent landslides during monsoon. Further loss in the natural habitat would completely wipe out the remaining plants in the particular habitat. Thus, we found that loss of habitat due to landslides and lacks of seed germination in nature were the major factors for the plant becoming critically endangered. Contamination was a major problem in raising the aseptic cultures of *Mantisia spathulata*. Fungal contamination mostly predominated than the bacteria. However, a particular bacterial pathogen repeatedly limited the establishment of *in vitro* aseptic cultures. Using sterilization procedure described earlier, only 20% of the cultures produced contamination-free rooted shoots that were subcultured regularly. After three weeks, 2-3 axillary shoots of 5-10 mm sizes sprouted from the nodal portion of the inoculated rhizomatous shoot explants on MS initiation medium supplemented with 8.9 μM BAP and 0.5 μM NAA (Fig. 2A). No shoot multiplication was observed in the control.

Multiplication of *in vitro* shoots and roots: Of the various concentrations of BAP and NAA tested for the plantlet production, neither of the growth regulators used singly and in combinations at higher concentrations was effective in the production of normal healthy plantlets. Explants treated with higher concentrations of BAP produced few, thick pale green, stunted shoots without roots in 4 weeks. On the other hand, numerous thick and small roots were obtained at higher concentrations of

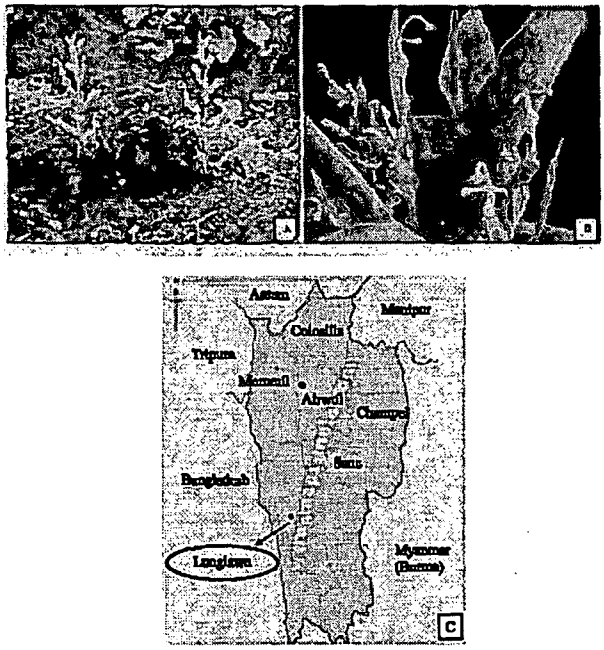

Fig. 1: (A) Flowering of *Mantisia spathulata* in the natural habitats after three years (B) A closed view of *Mantisia spathulata* flowers (C) Distribution of *Mantisia spathulata* in Mizoram

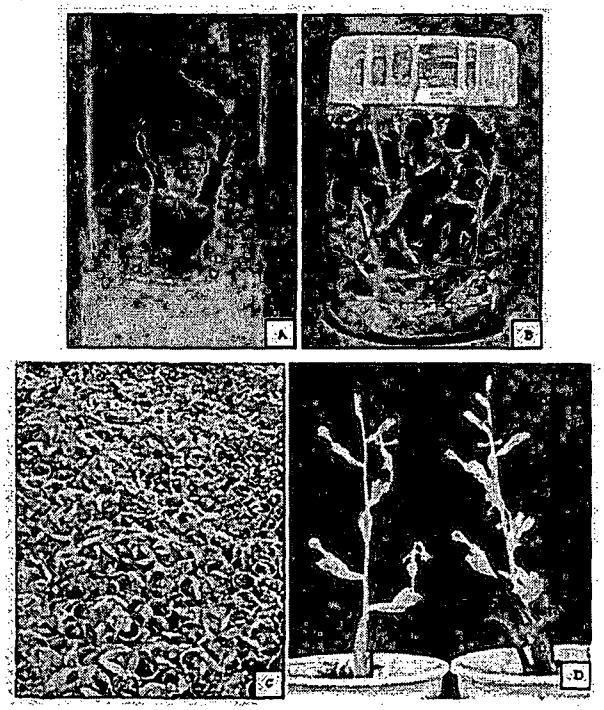

Fig. 2: Micropropagation of *Mantisia spathulata* (A) Shoot initiation on MS medium containing 8.9 μM BAP and 0.5 μM NAA after three weeks (B) Eight weeks old plantlets with multiple shoots and roots on MS medium containing 11.1 μM BAP and 2.7 μM NAA (C) Weaned plants after eight weeks of transfer (D) Flowering of morphologically invariant micropropagated plants under glass house condition after one year

Table 1: Effect of BAP and NAA on multiplication of *in vitro* plantlets of *M. spathulata*

Growth regulators (μM)		Mean No. of	Mean No. of
BAP	NAA	shoots/explant±SE	roots/explant±SE
0.0	0.0	0.4±0.21	1.2±0.33
2.2	2.7	2.4±0.66	6.0±1.16
11.1	2.7	5.2±0.95	8.6±0.82
22.2	2.7	4.2±1.03	6.4±0.66
44.4	2.7	1.4±0.53	4.6±0.72
LSD ($p<0.05$)		2.4	2.6
ANOVA		$F_{4,20}=5.62$*	$F_{4,20}=9.52$*

*Significant at $p<0.05$; ±SE (Standard error)

NAA which turned into callus after 10 weeks. Sprouting of shoots and roots occurred effectively in media containing a low concentration of NAA (2.7 μM) with all other concentrations of BAP (Table 1). Similar results were reported earlier for *Alipinia galanga* (Borthakur *et al.*, 1998) and *Kaempferia galanga* (Shirin *et al.*, 2000). Thus separate rooting medium as reported in other Zingiberous members like *Curcuma longa* Linn (Nadgauda *et al.*, 1978) and *Zingiber officinale* Rosc (Sharma and Singh, 1997) was not required. An optimum number of shoots (5.2±0.95) and roots (8.6±0.82) per explant was obtained in media containing 11.1 μM BAP and 2.7 μM of NAA. These are significantly higher ($p<0.05$) compared to those obtained at other concentrations and developed into normal healthy plantlets in 8 weeks. It was observed that 3 weeks old shoot buds (5-10 mm) before producing any roots if subcultured regularly at the same time interval maintains a high multiplication ratio. A single rhizomatous shoot produced 5.0 shoots within 3 weeks. Successive multiplication produced 9 shoots in the second subculture, 11 in the third and 8 in the fourth and fifth subculture, respectively. Thus, a single rhizomatous bud subcultured regularly at 3 weeks intervals can produce about 220 plantlets in 18 weeks which enables the rapid multiplication of *Mantisia spathulata*.

Weaning and transplantation: Plantlets of 100-150 mm size shoots were obtained within a short period of 8 weeks (Fig. 2B) without subculture; hence, expense for producing the plantlets was less. An initial weaning period of one week at 25±2°C with 80% RH followed by another week at 30±2°C at 60% RH in glass house was found to be significant for the higher survivability of the plantlets. About 90-93% of the plantlets were successfully weaned within 8 weeks (Fig. 2C). The weaned plantlets when transferred to bigger pots could grow as fast as the *ex situ* plants within 4 weeks. One year old weaned plants flowered in the glass house without any morphological variation (Fig. 2D). So far, more than one thousand morphologically invariant micropropagated plants have been developed and successfully reintroduced in their natural habitat. To the best of authors' knowledge, this is the first report, which describes the resurvey, collection and successful recovery of rare and endangered *M. spathulata* through our micropropagation protocol.

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- Participated in 3 day training course on "Data creation with emphasis on Biodiversity" through Bioinformatics tools (Funded by Department of Biotechnology, GOI): 22nd – 24th Feb 2005
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- Presented paper in National seminar on "Agricultural research in the North-east India" ICAR, Shillong: 10 – 12 Nov, 1999

Papers published : 1. Rapid *in vitro* clonal propagation of *Mantisia spathulata* Schult, A rare and endemic plant of North-eastern India for recover. **Biotechnology**. 6(1): 68 - 71, 2007

2. High frequency plantlet regeneration from rhizomatous buds in *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer and analysis of genetic uniformity using RAPD markers, **Indian Journal of Experimental Biology (IF 0.55)**. 47: 140 - 146, 2009

Communicated papers : **1.** *Ex situ* conservation of *Mantisa spathulata* Schult. and *Mantisia wengeri* Fischer, two critically endangered and endemic zingibers of Northeast India through seed germination. **J of Hort Sci and Biotech** (IF 0.6)

Significant achievements: Conserved around 2000 plantlets of two critically endangered *Mantisia* species in the laboratory garden from just two plants collected from the wild

Project during M.Sc : Title "**A study on fermented mustard seed Kharoli and preliminary investigation on starter culture development"**

Project during B.Sc : Title **"Low cost micropropagation of *Begonia rex* using absorbent cotton fiber as an artificial substrate".**

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