TISSUE CULTURE

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Plants are a valuable environmental and economic resource for supporting natural system and for improving human welfare. Everyone has benefited when people have treated forests as renewable resources, protected them to preserve biodiversity, or transformed them to support economic activities on a sustainable basis. On the other hand, destructive exploitation of the forests has caused serious economic, social, and environment losses, Hence, the need for conservation of phytodiversity cannot be overemphasised for the survival of mankind.

It is estimated that there are about 17,500 flowering plants in India. However, the ever increasing human population of our country and the various anthropogenic activities, coupled with natural calamities,have resulted into loss of habitat, thereby threatening a number of taxa with extinction. The gravity of the situation in India can be assessed by the fact that approximately 15-20% of the 17,500 flowering plant species are of concern, and possibly many of these may be lost in the next few decades unless proper attention is given for their conservation. It is feared that many of the species may be lost without being utilised or, even worst, before they are known to science.

Today, global efforts are being made to check the alarming erosion of phytodiversity. Two main approaches being used to conserve phytodiversity losses are *in situ* and *ex situ* conservation. *In situ* conservation is of primary importance for maintaining the broadest range of plant diversity. As a supplement to this approach *ex situ* conservation plays an important role in backing up taxa which are particularly threatened or are rare in the wild. Traditionally, botanical gardens, arboreta, seed and spore banks provided a valuable safeguard against loss of many rare species (Laliberte, 1997). However, in recent years, tissue culture (*in vitro* culture) offers the potential of extending these traditional *ex situ* conservation and propagation methods to an even broader range of taxa and tissue types. These techniques have been developed primarily for agricultural and horticultural species, but are increasingly being applied to propagating and evaluating rare and endangered plant germplasm as well.

Plant Tissue Culture (*In vitro* culture)

The term "plant tissue culture" was a precise one in the early days when tissue culture was mostly carried out with excised tissues. However, today the term has come to cover a great diversity of culture methods, including embryo, organ, protoplast, and suspension culture. In a broad sense, plant tissue culture can be described as a set of techniques for growing plant tissue isolated from parent plants in a defined nutritional and controlled environment under aseptic conditions (Bonga & Von Aderkas, 1992).

History of *in vitro* propagation of rare and endangered plant species

International

The earliest programmes to use *in vitro* propagation methods for rare and endangered species was the Micropropagation Unit at the Royal Botanic Gardens, Kew, established in 1974. Since then, several countries around the world have adopted the technology and have used successfully for prorpagation of many rare, endangered and endemic plants. Laboratories in Australia, Spain, USA and Hawaii have propagated their endemic floras (Clemente, 1991; Dixon, 1994; Iriondo & Perez,1991a; Koob, 1993), while laboratories in England, Denmark, Spain and elsewhere have also directed attention to propagating the endemic flora of islands such as St. Helena, Gran Canaria, and Rodriques (Fay, 1992; 1989; Krogstrup *et al.*, 1990; Ramsay, 1997). Other programmes around the world have applied *in vitro* propagation techniques to a wide variety of native and exotic endangered species. Several reviews have been published on the use of *in vitro* micropropagation of rare and endangered plants (Fay, 1992, 1994; Fay & Gratton, 1992; Wochok, 1981; Pence, 1999).

National

The Ministry of Environment and Forests, Governmentof India, realizing the importance of conservation of Indian flora, initiated a major All India Co-ordinated Project on Conservation of Plants in1985. This programme dealt with the seed biology and tissue culture of rare and endangered plants. Several Universities and National Laboratories have participated in this programme. National Bureau of Plant Genetic Resources (NBPGR) established the National Facility for Plant Tissue Culture Repository (NFPTCR) at its headquarters located at Pusa Campus, New

Delhi in 1986 with funding from the Department of Biotechnology, Government of India (Anon, 1999). Other important centres engaged in *in vitro* conservation of plants are Indian Institute of Spices Research,Calicut; Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow; National Botanical Research Institute (NBRI), Lucknow; National Chemical Laboratory (NCL), Pune; Gobind Ballabh Pant Institute of Himalayan Environment and Development (GBPIHED), Almora; Tropical Botanical Garden and Research Institute (TBGRI), Thiruvanantpuram; M.S. Swaminathan Research Foundation, Madras; Regional Plant Resources Centre (RPRC), Bhubaneshwar; Indian Council of Forestry Research and Education (ICFRE) Dehra Dun; Botanical Survey of India BSI, Eastern Circle, Shillong and several Universities in the country. The Department of Biotechnology, Government of India has given tremendous impetus to conservation of plants using biotechnological approaches by establishing two micropropagation Technology Park, one each at NCL, Pune and Tata Energy Research Institute, New Delhi; 4 hardening facilities for tissue cultured plants at J.N. Vyas University; RPRC, Bhubaneshwar and Kolkata, and GBPIHED; 4 national gene banks of medicinal and aromatic plants at CIMAP, TBGRI, NBPGR and Regional Research Laboratory, Jammu and also by funding a large number of R & D projects. As a result of the Government of India's initiative *in vitro* propagation techniques have been used successfully for a number of rare and endangered species by different research institutions and universities. Some such examples of work in India are shown in table 1.

Objectives of *in vitro* propagation of endangered species

The objectives of *in vitro* propagation of rare and endangered species are as follows:

(i) The primary objective for which *in vitro* propagation of endangered plant species is undertaken is to increase the numbers of individual species of extremely rare and endangered species. For example, *Sophora toromiro* an endangered species which is extinct in the wild, and the few plants which have been maintained *ex situ* have been used for *in vitro* multiplication (Iturriaga *et al.*, 1994). If wild population becomes severely reduced or lost, *in vitro* propagated plants can be used for reintroduction. *Rubus humulifolius*, an endangered species of Findland, known from ten plants, was propagated *in vitro* to yield 1500 plants and was replanted in a site near its original locality (Tormala *et al.*, 1994). Several rare species including *Agave victoria-reginae*, *Artemisia granatensis*, *Bletia urbana*,

Table I
Use of tissue culture for conservation of endangered plant species in India.

Family	Botanical name	Causes Status	Propagation methods	References
Acanthaceae	*Adhatoda beddomei*	Over-coll., med., few seeds, and slow prop.	Shoot-tips	Sudha and Seeni (1994)
Apocynaceae	*Rauvolfia micrantha*	Poor germ., poor rooting and med.	Shoot tips, nodes	Sudha and Seeni (1996)
	Rauvolfia serpentina	Over-coll., med. and poor seed variability.	Nodes - microprop.	Sharma and Chandel (1992)
	Wrightia tomentosa	Over-coll.	Nodes	Purohit *et al.* (1994)
Aquifoliaceae	*Ilex khasiana*	Rare and endangered	Young leaves	Tandon and Kumaria (1997)
Aristolochiaceae	*Aristolochia indica*	Over-coll. and med.	Shoot-tip and nodes, leaf, adv. shoot.	Manjula *et al.* (1997)
Asclepiadaceae	*Holostemma annulare*	Rare and med.	Shoot tips, nodes	Sudha *et al.* (1998)
Asteraceae	*Saussurea costus*	Critically endangered and med.	Shoot tip microprop.	Arora and Bhojwani (1989);
Betulaceae	*Betula uber*	30 individuals	Buds	Vijaykumar *et al.* (1990)
Combretaceae	*Anogeissus rotundifolia*	Rare and endemic	IV germ. and microprop.	Singh and Shekhawat (1997)

Family	Botanical name	Causes Status	Propagation methods	References
Dioscoreaceae	*Trichopus zeylanicus*	Few seeds, slow seed maturation, hab. loss and med.	IV germ., microprop.	Krishnan *et al.* (1995)
Fabaceae	*Pterocarpus marsupium* *Trifolium stoloniferum*	Rare Two populations	IV germ. and microprop. Shoot tips	Das and Chatterjee (1993). Singh *et al.* (1988).
Gentianaceae	*Gentiana kurrooa* *Swertia chirayata*	Over-coll. and med. Rare	Shoot tips, nodes Seedling stem, callus - adv. shoot.	Sharma *et al.* (1993). Shrestha and Joshi (1992).
Lamiaceae	*Coleus forskohlii*	Over-coll., med.	Nodes	Sharma *et al.* (1991).
Liliaceae	*Allium tuberosum*	Over-coll.	Basal plate, adv. shoot.	Radhamani and Chandel (1992).
	Chlorophytum borivilianum	Over-coll., med. and seed germ. low.	Shoot bases, microprop., somatic embryo.	Purohit *et al.* (1994); Jain *et al.* (1997).
	Lilium mackliniae	Rare and end.	Bulb scale, bulblet segments, cotyledon, leaf and root.	Mao *et al.* (2000).
Lythraceae	*Woodfordia fruticosa*	Rare and med.	Shoot tips, nodes.	Krishnan and Seeni (1994).
Nepenthaceae	*Nepenthes khasiana*	Rare and med.	IV germ., microprop., shoot tips, nodes - microprop.	Latha and Seeni (1994); Seeni (1990); Rathore *et al.* (1991).

Family	Botanical name	Causes Status	Propagation methods	References
Nymphaeaceae	*Nymphaea tetragona*	Rare and end.	Rhizome segments, immature embryos, young leaves, nodal segments.	Tandon and Kumaria (1997).
Orchidaceae	*Aerides multiflorum*	Rare	Rudimentary embryos	Tandon and Kumaria (1997)
	Aerides vandarum (= *Vandopsis undulata*)	Rare	Rudimentary embryos	Tandon and Kumaria (1997)
	Bulbophyllum cosmosus	Rare	Rudimentary embryos	Tandon and Kumaria (1997)
	Cymbidium giganteum (= *C. iridioides)*	Rare	Rudimentary embryos, root tips, leaves.	Corrie and Tandon (1993)
	Dendrobium lindleyi	Rare	IV germ.	Kaur and Sarma (1997)
	Dendrobium wardianum	Rare	Shoot apices, root tips, leaves, young floral buds.	Sharma *et al.* (1992).
	Paphiopedilum spp.	Rare	Rudimentary embryos	Tandon and Kumaria (1997)
	Renanthera imschootiana	Over-coll., hab. loss, prop. for trade.	Leaf bases, adv. buds	Seeni and Latha (1992).
	Thunia alba	Rare and endangered	Rudimentary embryos	Tandon and Kumaria (1997)
	Sarcanthus pallidus	Rare and endangered	Rudimentary embryos	Tandon and Kumaria (1997)
	Vanda coerulea	Over-coll.	Leaf bases, adv. shoot.	Seeni (1990).
	Vanilla walkeriae	Prop. for preserv.	Nodes	Agarwal *et al.* (1992)

Family	Botanical name	Causes Status	Propagation methods	References
Papaveraceae	*Meconopsis paniculata*	Seed germ. low survival, and hab. loss.	Hypocotyl, cal., adv. shoots.	Sulaiman (1994).
	Meconopsis simplicifolia	Hab. loss and seedling mortality	Seedling explants - cal. - adv. shoots.	Sulaiman and Babu (1993).
Podophyllaceae	*Podophyllum hexandrum*	Over-coll. and med.	Shoot tips; Hypocotyl.	Arumugam and Bhojwani (1990); Nadeem *et al.* (2000).
Polygonaceae	*Rheum emodi*	Over-coll. and med.	Shoot tips.	Lal and Ahuja (1993).
Polypodiaceae	*Drynaria quercifolia*	End.fern.	Spores and rhizome	Hegde and D'Souza (1997).
Ranunculaceae	*Aconitum heterophyllum*	Over-coll. and med.	Shoot tips, microprop. leaf, petiole, cal., som. emb.	Giri *et al.* (1993).
	Delphinium malabaricum	Low seedset and seed dormancy.	Infl. nodes.	Agarwal *et al.* (1991).
	Coptis teeta	Rare and end.	Petiole, apical and axillary buds, rhizome segments, inflorescence stalk, hypocotyl.	Tandon and Rathore (1992).
Rutaceae	*Citrus assamensis* *Citrus indica*, *Citrus latipes*	Rare	Shoot tips, microprop.	Baruah *et al.* (1996).
	Feronia limonia	Rare and endangered.	Nodal explants, axillary bud.	Purohit and Kiran Tak (1992).

Family	Botanical name	Causes Status	Propagation methods	References
Scrophulariaceae	*Picrorhiza kurrooa*	Rare and med.	Shoot tips, nodes, microprop.	Lal *et al.* (1988). Upadhyay *et al.* (1989).
	Limnophila indica	Conserv.	Root tips.	Rao and Mohan Ram (1981)
Sterculiaceae	*Sterculia urens*	Over-coll.	IV germ., microprop.	Purohit and Dave (1996).
Velerianaceae	*Nardostachys jatamansi* (= *N. grandiflora*)	Over-coll. and med.	Petiole-cal., root, adv. shoot.	Mathur (1992).
	Valeriana wallichii	Rare and med.	Shoot tips, nodes, microprop.	Mathur *et al.* (1988).

Abbreviations:

Med = Medicinal value; Prop. = propagation; Over-coll.= over-collection; Germ.= germination; End.= endemic; Hab.= habitat; Preserv.= preservation; Conserv.= conservation; IV.= *in vitro*; Adv.= adventitious; Microprop.= micropropagation; Hypocotyl= hypocotyledon; Cal.= callus; Lf.= leaf; Sh.= shoot(s); Fl.= Flower; Som. emb.= Somatic embryos; Imm.= immature; Infl.= inflorescence.

Nepenthes khasiana, Mammillaria san-angelensis, Senecio hadrosomus, Cyanea pinnatifida and orchids (Clemente 1991; Tandon *et al.*, 1990; Martinez-Vazquez & Rubluo, 1989; Bramwell, 1990) have been multiplied *in vitro* and their reintroduction in natural habitats were attempted.

(ii) *In vitro* propagation can provide an alternate source of plants and alleviate pressure on wild populations when species have been over-collected by hobbyists or for medicine, food, or fragrance. Certain orchids, cacti, and wild flowers as well as a number of medicinal species have been propagated *in vitro* for this reason (Rubluo *et al.*, 1993).

(iii) When wild grown plants are difficult to propagate using traditional propagation methods, tissue culture techniques can be used for *ex situ* preservation in botanical gardens (Christenson, 1988; Pence *et al.*, 1997). The tissue culture lines themselves can be conserved for short-term and cryopreserved for long-term storage. Propagated plants might also be used for *ex situ* studies on the biology of endangered plant species. For example, the Center for Plant Conservation, in U.S.A., St. Louis, coordinates with different botanical gardens in that country to monitor and grow endangered species *ex situ* (Pence, 1999).

(iv) Tissue cultured endangered plant species can also be conserved *in vitro* for short and long terms.

In vitro propagation techniques

The variety of approaches used by different laboratories around the world reflects the flexibility of tissue culture techniques. However, the different techniques used by different laboratories for *in vitro* propagation can be grouped into two (a) propagation using seeds and (b) These approaches are discussed below with few examples given in the specific context of rare and endangered species conservation.

I. PROPAGATION

a. In vitro propagation using seeds

When seeds of endangered species are available, they are generally preferred for propagation in order to maintain the maximum genetic diversity. *In vitro* seed germination has been applied to a number of rare

orchids species, cacti and succulents, insectivorous plants and lilies by many laboratories (Boulay, 1995; Clayton *et al.*, 1990; Dixon & Keighery, 1992; Fay, 1992; Fay & Gratton, 1992; Rubluo *et al.*, 1993; Seeni & Latha, 1994; Simerda, 1990; Singh *et al.*, 1992, Tandon & Kumaria, 1997). Most endangered species produce seeds in some cases they are few in number, or they may be difficult to germinate. when very few seeds are *in vitro* germination is often used to produce sterile seedlings, which are then provide shoot tips and nodes as explants for micropropagation. This approach has been used for a number of species, including, *Gentiana lutea, Limonium* spp., (Martin & Perez 1995; Momcilovic *et al.*, 1997 and many others (Pence, 1999).

Embryo culture is useful when conventional procedures, such as stratification, fail to break seed dormancy or the rate of germination is very low. Some forms of dormancy are overcome by removing the seed coat, as with *Trochetiopsis* spp. from St. Helena (Fay, 1992). Growth regulators are also used to stimulate germination, as with Western Australian rushes. Alternatively, growth regulators may be used to stimulate direct somatic embryogenesis or shoot formation from the embryo tissue or to produce embryogenic callus as with *Podophyllum hexandrum* (Arumugan & Bhojwani, 1990).

In some cases, seeds have particular requirements for germination which are not met by conventional germination procedures. For example, *Pholisma sonarae*, an endangered parasitic plant of South-Western United States, requires the presence of host root tissue for germination, and it has not been possible to germinate the seeds *ex situ* (Pence, 1999). Other root parasites have also been successfully germinated *in vitro* (Okonkwo, 1966).

Similarly, seeds of a number of rare orchids have been asymbiotically germinted *in vitro,* such as *Vanilla alkeriae, Paphiopedilum* spp., *Dendrobium lindleyi,* etc. (Agarwal, *et al.* 1992; Kaur & Sarma, 1997; Tandon & Kumaria, 1997). In cases such as *Spiranthes magnicamporum,* symbiotic cultures of seeds and fungus have been established *in vitro* (Anderson, 1991). Germinated orchid seeds have been used to initiate cultures for micropropagation (Christenson, 1988).

b. Propagation without seeds

Propagation by seeds may not be possible for some endangered species. Seed viability can be low, as with *Rauvolfia micrantha* (Sudha & Seeni,

1996), etc., while in some cases little or no seed is produced, such as *Haworthia* spp., *Paronychia chartacea, Adhatodha beddomei* and *Delphinium malabaricum* (Agrawal *et al.*, 1991; McKently & Adams, 1994; Rogers, 1993; Sudha & Seeni, 1994). When seeds are not available, *in vitro* propagation is accomplished by culturing shoot tips or nodes from field or greenhouse grown plants. The culture of preformed meristems is preferred for propagation because of their genetic stability.

The growth habit of some species is such that the culture of apical or vegetative lateral buds would irreversibly damage the plant. In the case of monopodial orchids, such as *Phalaenopsis*, the culture of dormant buds from inflorescence nodes has been used to overcome this problem (Reisinger, *et al.*, 1976). In case of *Delphinium malabaricum* also inflorescence nodes have ben used, since the single apical bud also grows at soil level, making it difficult to establish uncontaminated cultures (Agrawal *et al.*, 1991).

Although the culture of preformed meristems is generally preferred, because of their genetic stability there are situations where buds are not available or difficult to culture or where more rapid propagation methods are desired. Organogenesis or embryogenesis has been obtained from vegetative tissues of *Meconopsis simplicifolia, Dionea muscipula, Agave victoria-reginae*, and *Haworthia* spp. (Kukulczanka *et al.*, 1989a; Rodriquez-Garay *et al.*, 1996; Rogers, 1993; Sulaiman, 1994). Species in the Liliaceae and Amaryllidaceae are often propagated using bulb-scales or similar tissue (Drewes & van Staden, 1994; Kukulczanka *et al.*, 1989b; Pandey *et al.*, 1992; Mao *et al.*, in press), where procorm-like bodies have been produced from leaf segments of monopodial orchids (Tanaka & Sakanishi, 1977). In the case of *Nardostachys jatamansi*, which naturally forms buds from its roots, petiols callus was used to initiate adventitious roots *in vitro*, which were then stimulated to form buds (Mathur, 1992).

d. Rapid clonal propagation

Tissue culture is a powerful tool for rapid clonal propagation of a particular genetic line. The technique has revolutionised the orchid industry. It may appear to contradict the goal of preserving genetic diversity. However, genetic diversity is maintained by culturing each individual available for propagation as a unique and separate line.

A related concern is that of the introduction of genetic changes or somaclonal variation into an otherwise clonal line. Generally, plants obtained from preformed buds have a lower frequency of change than those from direct adventitious sources, while those from callus appear most likely to undergo changes (Karp, 1994). However, a number of factors are involved in developing a protocol for propagating an endangered species, and at times, buds cannot be used. In those cases, it may be necessary to regenerate adventitious shoots, but it may then be possible to propagate those shoots by axillary bud outgrowth. Another approach can be to grow on a minimal level of growth regulators, in order to minimise the potential of somaclonal variation. This approach has been carried out with the micropropagation of the rare *Hackelia venusta* from the northern United States (Edson *et al.*, 1996).

Increasing genetic diversity

When plant are regenerated from calli or through somatic embryogenesis or by adventitious shoot formation, it often happens that a new genotype arises. This phenomenon is called somaclonal variation and occurs in many plants (Larkin & Scowcroft, 1981). There are many possible causes, one of which is the naturally occurring variation within the plant, but it is enhanced by artificial conditions during the tissue culture.

Somaclonal variation has been suggested as a tool for increasing the genetic diversity in species with a very narrow genetic base, such as the Easter Island endemic, *Sophora toromiro* (Jacobsen & Dohmen, 1990).

II. *IN VITRO* CONSERVATION

In vitro conservation has been proposed as a safer alternative to the traditional methods for preserving plant germplasm, *ex situ* have included growing plants in botanical gardens, arboreta and banking the dried seeds and spore at refrigerator (4 °C) or freezer (-18 or -20°C) temperatures (De Langhe, 1984; Withers, 1984, 1991). Considerable interest has been shown in recent years on the application of tissue culture technology for the storage of plant germplasm. The main prerequisite of *in vitro* conservation is satisfactory storage and, in parallel to the seed bank, there is a need for both 'active' and 'base' in vitro storage technologies (Withers, 1991). Some of the most practical applications of in vitro conservation relate to germplasm acquisition and movement. Currently two methods are being

used for *in vitro* conservation of plant germplasm. These are slow growth culture and cryopreservation.

Slow growth culture

For medium term storage of several months to several years, slow growth culture method is being used for preserving *in vitro* cultures of endangered species. Several strategies can be applied to slow the growth to maintain plant germplasm, for example: manipulating the basal medium, lowering the optimal nutrient levels; altering the physical conditions such as temperature, light regime and gas atmosphere; application to the medium of growth retardants (e.g. absicic acid) or osmoregulators (e,g. sorbitol, mannitol) (Dodds & Roberts, 1985). Most established slow-growth protocols have been applied to crop species (Dorion *et al.*, 1994; Benerjee & de Langhe, 1985) and few reports exists on the germplasm conservation of endangered plant species (lriondo & Perez, 1991). Good survival has been reported to shoots of *Centaurium riguali* for three years, *Picrorhiza kurroa* for ten months, *Saussurea costus* for 12 months, and of *Coronopus navasii*, *Lavatera longifolia*, and *Centauriuim rigualii* for six months, when stored at 5°C (Arora & Bhojwani, 1989; lriondo & Perez, 1996; Upadhyay, *et al.*, 1989). Similarly, *Drosera* spp. and *Dionaea muscipula* have been maintained for up to ten months at 0-6°C (Kukulczanka, 1991). *Rauvolfia serpentina* remained healthy after 15 months at 15°C, although lower temperatures were deleterious (Sharma & Chandel, 1992).

Cryopreservation

The development of cryopreservation, or storage in liquid nitrogen (at - 196°C), has provided a technology for long-term storage of living tissue. It has been developed as a suitable methods for long term germplasm conservation, through cessation of cell division, thus avoiding the possibility of genetic variation through *in vitro* cell division (Engelman, 1991). A wide variety of protocols have been developed for cryopreservation of vegetative material as well as seeds over the last two decades (George, 1993). Several laboratories have applied cryopreservation protocols to seeds of a variety of endangered species. In U.S.A., a co-operative agreement between the National Seed Storage Lab (NSSL) of the US Department of Agriculture and the Center for Plant Conservation was developed to store seeds of endangered US species at the NSSL facility (Falk, 1987), while seeds of endangered and threatened species of Ohio are cryopreserved at the Cincinnati Zoo and Botanical Garden (Pence,

1991). Cryopreservation is being applied to the seeds of the endangered and rare flora of Western Australia at Kings Park and Botanic Garden (Touchell & Dixon, 1994). In India, National Bareau of Plant Genetic Resources (NBPGR), New Delhi was developed to store seeds and tissue culture plant materials of economically important plant species of the country. Several other laboratories around the world have also developed cryogenic storage facilities for seeds and vegetative material of native flora.

However, the majority of species currently stored in liquid nitrogen are those with orthodox, or desication tolerant seeds. The dried, orthodox seeds generally survive liquid nitrogen exposure with little or no damage. In some cases, seeds may be orthodox and short-lived unless they are carefully dried and frozen either at -20°C or in liquid nitrogen. *Plantago cordata* and *Salix myricoides*, listed as endangered and potentially threatened in Ohio, are two examples of the short-lived seeds which have been successfully dried, cryopreserved and banked in liquid nitrogen (Pence, 1998).

Species having 'recalcitrant', seeds cannot usually survive drying and in the hydrated state they do not survive exposure to liquid nitrogen. However, cryopreservation is being increasingly applied to the excised embryos of recalcitrant seeds from tropical tree species. New approaches to recalcitrant seeds cryopreservation are presently being considered. In general, still recalcitrant seeds do pose particular problems for long-term germplasm storage. Seeds of some large-seeded temperate trees, some wetland species and some climax species from the moist tropics fall into this category. Wetlands and moist tropical forests are two habitats that are particularly threatened. There is an increasing need for *ex-situ* germplasm storage of species from these areas. Studies are underway at the National Seed Storage Laboratory (Ft. Collins, Colorado) to determine the extent of recalcitrance in seeds of endangered species from the rain forests of Hawaii and by the Cincinnati Zoo and Botanical Garden, species from Ohio wetlands, so that seed storage protocols can be developed for these species. It appears that in both cases, the majority of the species under study are not recalcitrant (Pence, 1999).

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. These procedures centre around the techniques of slow freezing (Withers, 1985), vitrification (Sakai *et al.*,

1990), and encapsulation-dehydration (Fabre & Dereuddre, 1990). Most commonly used tissue for cryogenic storage are: shoot tips from *in vitro* cultures, excised zygotic embryos and embryonic axes, somatic embryos and embryogenic or organogenic cell or callus lines (Pence, 1999). Cryopreservation of *in vitro* tissue from endangered species has been accomplished using a slow freezing protocol with shoot tips of *Grevillea scapigera* and organogenic callus of *Dioscorea caucasia* and *D. balcanica* (Chulafich *et al.*, 1994; Touchell *et al.*, 1992). Other species, which have been cryopreserved using encapsulation-dehydration, include *Centaurium rigualii*, endemic to the Iberian peninsula (Gonzalez-Benito & Perez, 1997), and *Cosmos atrosanguinensis*, which is cultivated but extinct in the wild (Wilkinson *et al.*, 1998).

Spores and gametophytes of pteridophytes and bryophytes can also be cryopreserved. Non-chlorophyllous fern spores are generally desiccation tolerant and adapt well to liquid nitrogen (LN) storage, although some are short-lived, such as those of the endangered tree fern, *Cyathea spinulosa*. These were dried and exposed to LN, with over 93 per cent recovery (Agrawal *et al.*, 1993). Chlorophyllous spores of at least some ferns, though generally short-lived, can also be dried and cryopreserved or cryopreserved using the encapsulation-dehydration technique (Pence, 1999).

Some gametophytes of mosses and liverworts are by nature desiccation tolerant and when this is the case, they can be air dried and frozen directly in liquid nitrogen (Leverone and Pence, 1993). When gametophytes are sensitive to drying, pre-culture with abscisic acid (ABA) is sufficient to induce desiccation tolerance in some species of bryophytes. In other cases the encapsulation-dehydration technique has been useful in preserving both tropical and temperate bryophytes and fern gametophytes through disiccation and subsequent liquid nitrogen exposure (Pence, 1999). Slow freezing has also been used to cryopreserve protoplasts of *Marchiantia* (Takeuchi *et al.*, 1980), while pre-culture with mannitol or ABA and proline has been shown to provide protection of moss tissues through slow freezing protocols (Christianson, 1998; Grimsly and Withers, 1983). These techniques should be readily transferable to rare or endangered bryophytes and pteridophytes.

However, cryopreservation technique necessitates specific technical equipment, not readily available in many laboratories in the developing countries. Hence, the use of slow-growth conditions by lowering the temperature is more commonly employed.

c. *In vitro* collection

In vitro collection, or IVC, is the initiation of tissue cultures in the field. It can be used to collect germplasm of species for which seeds are not available and for which cutting may be difficult to maintain or transport. IVC is a very flexible technique and can be adapted to a variety of situations. Either partial or full sterilization of the tissue is made on site and the tissue is transferred to containers of medium for transporting back to the lab. For example, tissues of *Cocos nucifera* have been collected with minimal treatments in the field (Assay Bah *et al.*, 1987). Once they were transported back to the lab, they were resterilized and dissected further for culture. In other cases, sterilization and dissection have been completed in the field, the growth and development of the cultures initiated at the point (Pence, 1996). IVC has been used to collect a variety of plant tissues, including orchid seeds (Warren, 1983), embryos (Assy Bah *et al.*, 1987), apical or nodal buds (Ruredzo, 1991; Yidana *et al.*, 1987), and leaf and stem tissue (Pence, 1996).

Different strategies have been used to minimize contamination in IVC cultures. In some cases, a portable glove box was used to reduce contamination from ambient sources (Sossou *et al.*, 1987), whereas in other cases the work has been done quickly in the open air. Internal contamination can be a more serious problem than ambient contamination, since many plants harbour endophytic fungi and bacteria. However, the use of fungicides and antibiotics in the medium can reduce this contamination to a workable level (Pence, 1996).

The intiation of *in vitro* cultures in the field can facilitate the transport of the tissues. Because of the small size of the explants, more material can be transported, compared to the transport of whole plants or cuttings. In addition, the cultures are initiated with fresh material which can begin the process of growth *in vitro* immediately, comparaed with whole plant materials which may undergo some deterioration in transport before they are planted *ex situ*. Finally, the transport of clean plant materials *in vitro* generally facilitates their movement through international border inspections.

IVC can be used as a source of material for both the propagation and preservation of endangered plant germplasm. For example, leaf and bud tissue collected by IVC from *Brunfelsia densifolia*, a rare Puerto Rican tree growing at the Fairchild Tropical Garden in Florida, was transported

to the Cincinnati Zoo and Botanical Garden where plants were regenerated from the cultures and tissues were cryopreserved (Pence, 1990).

The limitation of IVC are those of tissue culture in general, since condition for growing some species *in vitro* have not yet been developed. However, the number and variety of species which have been successfuly propagated *in vitro* continues to grow, and this will, in turn, be reflected in the widening applicability of IVC techniques for the collection of rare or endangered plant germplasm.

Application of tissue culture in conservation in India

Application of tissue culture in *ex situ in vitro* conservation in India has been more or less restricted to economically important plant species which have become rare and endangered due to over-exploitation (see Table-1). It is also observed that most of the work done are on rapid mass multiplication and less on *in vitro* conservation either for short or long term conservation. Most of the work carried out are done in universities and they end up there when the project is over. Thus, the work is not carry over or the result is not being utilised by concern institutions or departments. A co-ordinated research and development is therefore, needed in rare and endangered plant species regardkess of its economic status, so that the finding of the research is properly utilised.

In India, the funding for R & D in the area of rare and endangered genetic resource conservation is also still very low and an area very much neglected when compare to economically important crop species. Some rare species may be relatively unknown, but could have horticultural or other value if enough materials is made available for breeding and development. Hence, the funding must be enhanced in order to carry out the work effectively.

More research on *in vitro* methods for multiplication and conservation have been conducted on orchids than any other group of plants in India. Sharma *et al.*, (1993) have presented a brief review of the orchid research work caried out by different workers in India. Currently, application of tissue culture techniques to conserve and commercialise Indian orchids is being assessed at Panjab University, Chandigarh, Botanical Survey of India, Shillong, North Eastern Hills University, Shillong; Orchid Research and Development Centre, Tippi, Arunachal Pradesh; Indian Institute of Horticultural Research, Bangalore and Tropical Botanic Garden and

Research Institute, Thiruvananthapuram. Attempts are being made at NBPGR, New Delhi, to conserve recalcitrant seeds of mango, coconut, jackfruit, litchi, sapota, walnut and other economic plants. Considerable success in cryopreservation of economic plant germplasm has been made at National Facility for Plant Tissue Culture Repository (NFPTCR) using seeds, pollen and *in vitro* cultures (Chudhury *et al.*, 1989). Successful cryopreservation, using desiccation of embryonic axes followed by rapid freezing, was achieved in tea, jackfruit, trifoliate orange, and almond. Effects of cryopreservation on seed germination of selected rare medicinal plants of India are being studied at Tropical Botanic Garden and Research Institute, Thiruvananthapuram.

Limitation of tissue culture in conservation of endangered species

The limited amount of plant material available is the controlling factor in tissue culture of rare endangered species. Generally, work with related, non-endangered species is used as a guide, therefore, the ability to test protocols and even to conduct replicated experiments may be severely limited (Campos & Pais, 1996; McComb, 1985). In addition, plants may be located in remote or difficult to access areas and collecting trips may be expensive. Permits are often required for any collection, as well as for transport. Also, the financial resources available for work with endangered species is often limited in comparison to that of economically important species. However, despite these limitations, a significant amount of work has been done with endangered plant species to solve specific problems in the areas of propagation, germplasm preservation, collection and analysis of genetic diversity (Pence, 1999).

CONCLUSION

As already mentioned in the text, a wide range of endangered plants has been sucessfully propagated in many countries using *in vitro* techniques. This has faciliated easy distribution of material of these species to other institutions around the world because of the cultures do not require quarantine due to their sterile nature. *In vitro* propagation has also allowed materials to be stored in *in vitro* gene banks, and this will increase with further developments in cryopreservation technology. However, in spite of these substantial advantages over conventional methods, there are certain inherent limitations which should be borne in mind. It is now well established that frequent genetic modifications are manifested as heritable

mutations among the progeny of regenerated plants. This phenomenon is called somaclonal variation and is defined as genetic variability generated during tissue culture (Larkin & Scowcroft, 1981). This variations has obvious benefits as an adjunct to plant improvement but it poses serious problems for *in virtro* germplasm conservation and exchange.

Nevertheless, an increasing number of botanic gardens around the world have *in vitro* facilities and information on techniques is disseminated among botanic gardens. A newsletter called *Botanic Gardens Micropropagation News* was launched in 1990 and published by Royal Botanic Gardens, Kew, (U.K.) in association with Botanic Gardens Conservation International. This issue is also available on the World Wide Web:http://www.rbgkew.org.uk/science/micropropagation/bgmnews.html.

Apart from the above mentioned *in vitro* techniques, the greatest challenge of the millenium is the development of molecular techniques. Molecular technique has opened the door to a number of areas relevant to conservation, management and monitoring the stablity and diversity of endangered plant germplasm held *in situ* and *ex situ*. Molecular techniques, particularly RAPD analyses, are being used to monitor the genetic diversity of populations of rare species, as well as to define species themselves which is very important for endangered species management. For example, a high level of diversity was found for the ten known populations of *Banhsia cuneata*, an endangered species of South-western Australia (Maguire & Sesgley, 1997). RAPD analysis also indicated that the one known individual of *Eucalyptus graniticola* was a hybrid of two more common species, rather than an endangered relict species (Rosetto *et al.*, 1997). Thus, rather than reinforcing the population *in situ*, the species was backed up *ex situ*. The use of RAPDs can provide an even more precise tool for the detection of somaclonal variation in micropropagated endangered plants (Martin & Perez, 1994).

In preserving plant germplasm, DNA can be banked as a back-up or supplement to the storage of living tissues, or may be used when living tissues cannot be stored. Techniques for isolating DNA from dried tissue, as well as dried frozen material have been developed (Adams & Adams 1992), making collection and banking from wild, remote species a posibility. Libraries of DNA from rare or endangered species are being set up in many laboratories around the world to store this information for future use (Mattick *et al.*, 1992). Also, recommendations and guidelines for DNA banking have been made (Adams & Adams, 1992).

Further, molecular technologies offer the possibility of collecting ancient DNA from extinct species, as well. Within the past decade, the isolation of DNA fragments from dried herbarium specimens (Pyle & Adams, 1989) as well as from fossil materials (Rogers & Bendich, 1985; Suyama *et al.*, 1996) has been demonstrated. A fragment of DNA, identified as part of the RuBisCo gene, was obtained from a fossil leaf compression of the extinct *Magnolia ltahensis* (Golenberg *et al.*, 1990). This was compared with gene sequences from extant species. A portion of this same gene was also obtained from fossil *Taxodium* Soltis *et al.*, 1992).

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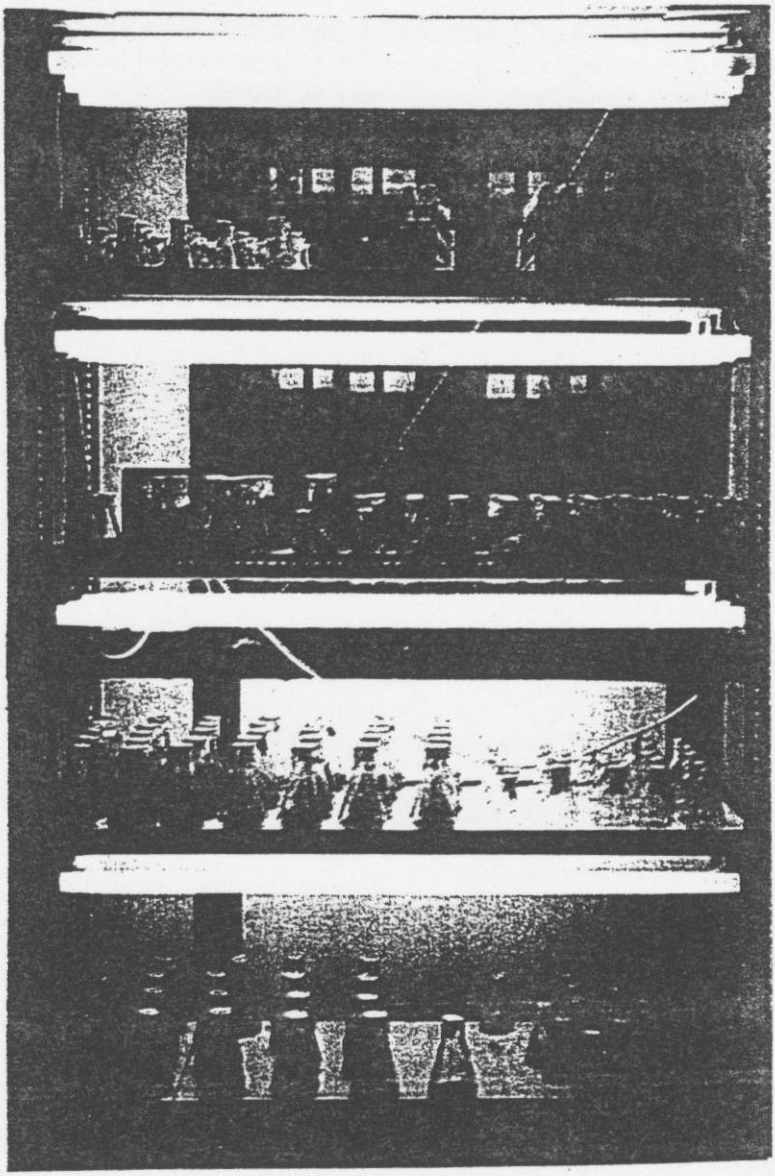
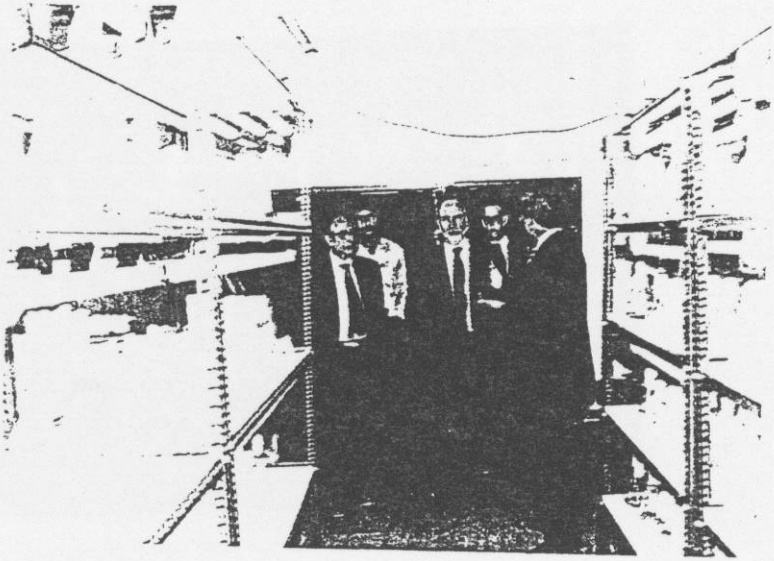


A view of Tissue culture Laboratory, BSI, Shillong.



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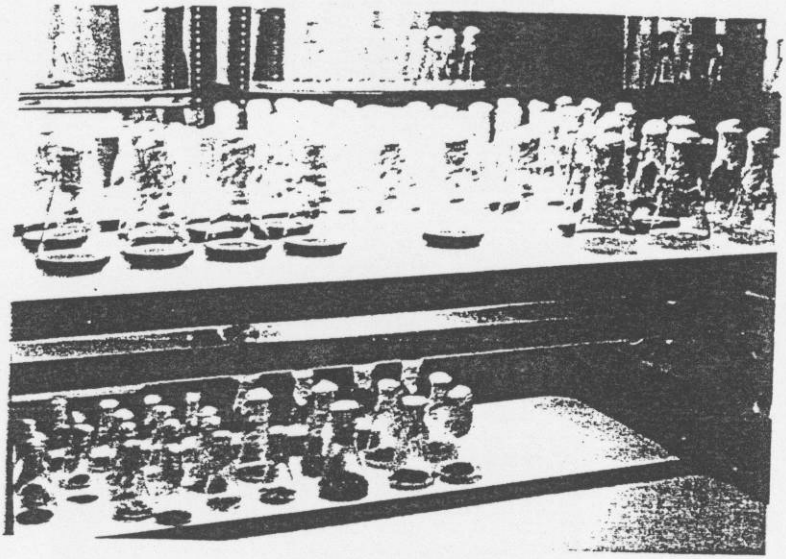
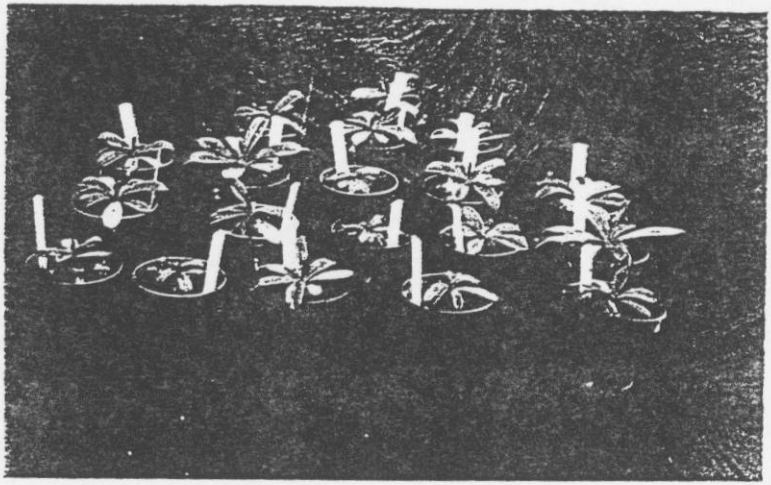
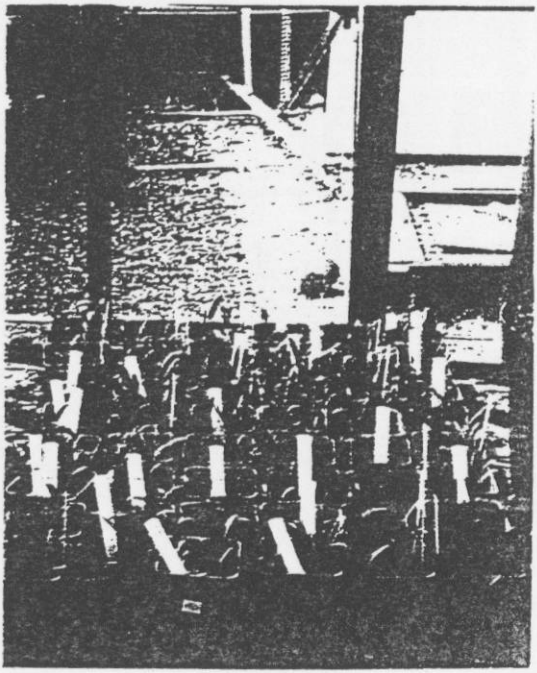


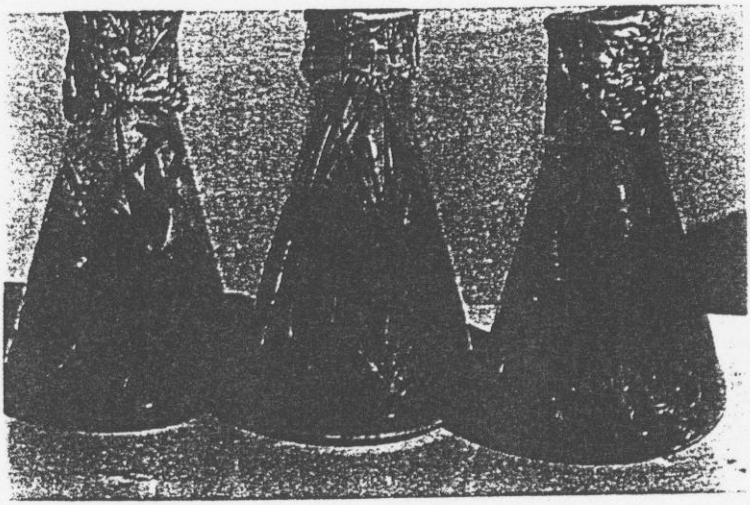
The same in closer view.



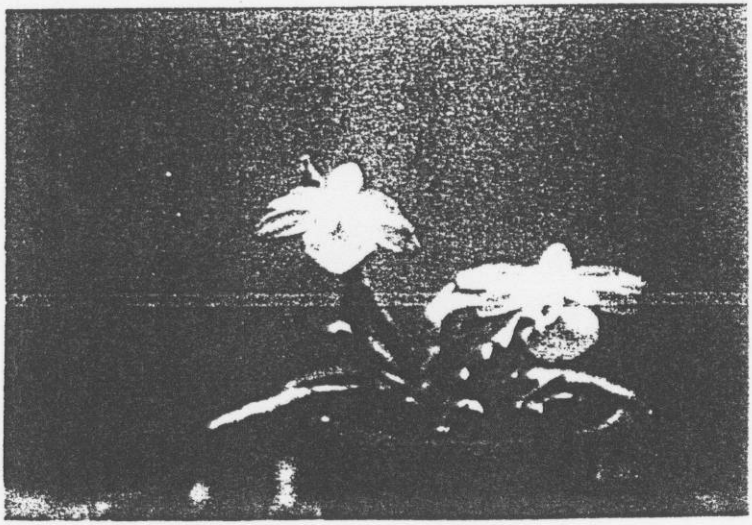
Nepenthes khasiana - *in vitro* raised plants.



Lilium mackliniae - weaned plantlets.



L to R : Culture of *Dendrobium nobile*, *Arundina graminifolia* and *Cymbidium tracyanum*.



Successfully transferred lab to land *Dendrobium primulinum* in flower.