ROLE OF BIOTECHNOLOGY IN CONSERVATION OF PLANT GENETIC RESOURCES IN THE 21 ST CENTURY -AN INDIAN PERSPECTIVE

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India is recognized as one of the world's mega-diversity countries with two 'hot-spots' namely, the Western Ghats and Eastern Himalayas. The mosaic of geo-climatic conditions influences the rich floristic wealth of the country. A number of plants are considered to have originated here. Due to unplanned human activities and natural calamities the plant genetic resources are getting depleted at an alarming rate. Biotechnological interventions namely, molecular marker technology, *in vitro* propagation and establishment, short-to medium term storage and cryopreservation play a significant role in conservation of plant genetic resources.

Introduction

India is rich in flora and possesses a great variation in vegetation (Nayar, 1997; Rao, 1997). Currently available data place India in the tenth position in the world and fourth, in Asia in plant diversity (Anon., 1999a). Despite concerted efforts of scientists of Botanical Survey of India, Universities and National laboratories, only 70% of the geographical area have been surveyed so far. The forest wealth, including tropical forests, is getting depleted in millions of hectares each year in some of the most diverse ecosystems in the world. With shrinking habitats due to population explosion and unplanned human activities the vegetation is declining sharply. There is an increasing pressure on the plant genetic resources for human survival and economic well being through out the world. There has been extinction of many plant species. Use of high yielding crop varieties by replacing traditional land races has threatened the diversity in crop plants. The most-often asked questions are 'What diversity does India possess?', 'What to conserve?', and 'How to conserve?'. Traditional approaches to germ plasm storage in seed and field gene banks have contributed to the secure conservation of gene pools, as do *in situ* and on-farm conservation. However, these approaches have some drawbacks. In recent years efforts have been made to develop new conservation methods based on biotechnology. The complementary application of these approaches along with the traditional conservation methods has made an impact. Sharma (1999) has eloquently described

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the opportunities and challenges and the role of biotechnology in biodiversity characterization, conservation and its sustainable use. India is keen to implement principles of Biological Diversity Conservation, but there is a need for formulating our own biodiversity protocol and update its regulations pertaining to biotechnology. Wide ranges of biotechnological tools are now utilized (including tissue culture techniques, molecular genome analysis, immunological diagnostics and cryopreservation protocols) for the collection, characterization, disease indexing, propagation, patenting, storage, documentation and exchange of plant genetic resources. This presentation aims at describing some of these aspects in general and *in vitro* propagation and conservation of plant genetic resources in particular.

The Indian Gene Centre

India is one of the world's top 12 'mega-diversity' countries. The Eastern Himalayas and Western Ghats are among the 18 'hot-spots' identified in the world. The Indian mainland extends between 8°4' to 37°6' N and 68°7' to 97°25' E and has a land frontier of about 15,200 km and a coastline of over 7516 km long. The country has an immense array of environmental conditions ranging from the extremely cold snow clad mountains in the north to a very hot area in the Thar desert having virtually no rainfall to one of the world's wettest places in the North-east. Consequently, India has enormous variation in the vegetation pattern, an attribute further enhanced by the meeting of three major biogeographic realms, namely, the Indo-Malayan (the richest in the world), the Eurasian and the Afro-Tropical. The Indian region falls into two biogeographical realms, viz., Palaearctic and Indo-Malayan (Udvardy, 1975) and has 10 biogeographical provinces -Himalayan highlands, Thar desert, Malabar rain forests, Bengalian rain forest, Indo-Ganges monsoon forest, Mahanandian, Coromandel, Deccan thorn forest, Laccadive islands and Andaman and Nicobar islands.

India is very rich in the multiplicity of the life forms, which exist on the planet. This is also true for the diversity of ecosystems, species and diversity of the genetic pool within the species. The plant wealth of India is estimated to be over 49,000 species, which is 12% of the known plant species of the world. The flowering plants of India comprise about 17,000 taxa, of which 5725 are broadly considered endemic and represent 33.5% of the flora located in 26 endemic centres. The number of endemics in the three geomorphological areas is i) Himalayan taxa: 3471; ii) Peninsular Indian taxa: 2015; and iii) Andaman and Nicobar taxa: 239. There are two mega-centres of endemism in India: the Eastern Himalayas, 1808 spp; and Western Ghats, 1500 spp (Nayar, 1997). The number of endemic plants may be still higher owing to the fact that these also include the plants of medicinal and religio-

ritual values, which were semi-domesticated to semi-wild. There are about 64 species of gymnosperms, 1022 of pteridophytes, 2584 of bryophytes, 1600 of lichens, 23000 of fungi, 2500 of algae, and 850 of bacteria. Nearly 2,560 (17%) species of the Indian flora are tree species and approximately half of the world's aquatic flowering plants are known to occur in the Indian sub-continent (Rao, 1997).

A large number of crop plants (348) are cultivated in India. The Indian region alone has contributed to the world nearly 168 economic plants whose centre of origin/ diversity lies in India along with their wild relatives (326 species) and land races such as cereals, millets, legumes, vegetables, fruits, forages, fibres, sugar-yielding types, spices, condiments, medicinal and aromatic plants and others grown in the diverse phytogeographical/ agro-ecological regions. The Western Ghats, North-eastern Himalayas, Southern plateau, Central India and North-western Himalayas possess greater genetic diversity comprising native species and land races than that in the other regions of the country.

The Indian crops showing a rich diversity are rice, wheat, barley, pigeonpea, chickpea, minor-millets, mungbean, uradbean, horsegram, mothbean, ricebean, clusterbean, sesame, forage grasses, okra, eggplant, cucumber, melons, citrus, banana and plantains, jackfruit, mango, tamarind, jamun, jute, cotton, ginger, turmeric, pepper, cinnamon and cardamom. A rich diversity is reported in sweet potato, taros and yams. Several plant species exhibit native resources for example, Coleus, sword bean, velvet bean and plantation crops including arecanut and coconut. Several minor fruits also possess diversity, e.g., berries and nuts, *Rubus*, *Ribes*, *Juglans*, *Pyrus*, and *Prunus* (Paroda *et. al.*, 1999).

The Indian region also boasts of a rich diversity in orchids, rhododendrons, bamboos, balsams and primulas. Bamboos cover about 13% of the forested area, which has played an important role in the economy of the country from ancient times. There are 18 genera and 130 species of bamboos known from India, of which 15 genera and 63 species are reported to occur in northeastern India. This region also exhibits genetic diversity for the species of *Bambusa*, *Dendrocalamus* and *Arundinaria*. Rhododendrons, famous for their beautiful flowers, are represented by 90 species in India, of which 80 are found in Eastern Himalayas alone. The orchids bearing splendid flowers are represented by 163 genera and 1100 species. About 780 species occur in northeast India and Eastern Himalayas. There are 179 genera and 1152 species of legumes in India, of which 263 taxa are endemic. *Crotolaria* (96 spp), *Astragalus* (72 spp), *Acacia* (70 spp), *Indigofera* (60 spp) and *Dalbergia* (35 spp) are dominant genera of legumes. Nearly 7,500 species of plants are used in India for medicinal purposes (Natesh, 1999). The Himalayan region is very rich in medicinal plants. Some of the important medicinal plants are: *Acorus*

calamus, *Costus speciosus*, *Dioscorea spp*, *Hydnocarpus kurzii*, *Gloriosa superba*, *Rauvolfia serpentina*, *Coptis teeta*, *Gentiana kurroa*, *Nardostachys jatamansi*, *Picnorhiza kurroa*, *Podophyllum hexandrum*, *Aconitum* spp, *Ephedra gerardiana*, *Rheum emodi*, *Digitalis purpurea*, *Taxus baccata*, etc.

Under the Indian Wild Life (Protection) Act, 1972, the Ministry of Environment & Forests has set up 85 national parks and 448 sanctuaries and reserves with a total area of 10 million hectares or about 4.5% of the area of the country. In addition, 14 biosphere reserves have been identified, of which 9 have been established (Anon., 1997).

Significance of Plant Genetic Resources and Need for their Conservation

For more than 10,000 years plants have been used for agriculture and still provide the genetic base for new varieties of crops. The Indian economy is predominantly agriculture based. About 93% of the human foodstuffs are products of plant origin. Plant genetic resources are the very basis of the human survival and economic well being as they provide food, clothing, shelter, medicine, biomass energy and industrial raw material, which offers a potential for providing many more yet unknown benefits to the future generation. It has been realised that valuable and productive plant germplasm resources are crucial for sustainable economic development. The rural people in India have always known that biological resources of crops and forests mean their survival. Between 70- 80% of the population relies on plants as the only source of medicine and over 7000 plants (out of 8900 species of ethnobotanical interest) are known to be used for medicinal purposes in the country.

The conservation of plant genetic resources has been long realised las an integral part of plant genetic improvement programmes. In the absence of genetic diversity it would not have been possible to make the genetic advances seen in crops worldwide during the last century. The 'Green Revolution' was mainly possible due to our ability to harness the benefits of plant breeding, crop physiology and germplasm collections of important crop plants such as wheat, rice and maize.

Cultivated crops are inbred for uniform growth and flowering patterns, yield and other desirable traits. This narrow genetic base caused many disasters in crop plants. The potato famine of 1846 in Ireland was due to the fact that no genetic diversity in Irish potato was available and it was multiplied using a clonal material with no resistance to the 'late blight' fungus, *Phytopthora infestans*. Another example is the epidemic of the southern corn 'leaf blight' caused by the fungus, *Helminthosporium maydis* in the USA during 1970. This corn was mostly of hybrid origin and possessed the 'Texas cytoplasmic male sterile line'. The fields of corn

had an unlimited extremely narrow gene base habitat for the fungus. It was only through other corn genetic resources that resistance was developed in the hybrid corn (Adams, 1997).

Biodiversity generates economic value in different ways. It has been a source of revenue from extractable products obtained from individual species. The use of compounds, genes and species is essential to meet the industry needs. Recently, greater attention has been paid to bioprospecting that links biodiversity and industry. In the past, this activity generated revenue mostly for the industry and hardly any efforts were made for biodiversity conservation and flow of profits to the source countries (Tamayo *et al*, 1997). Protection of plant genetic resources against biopiracy needs utmost priority and patenting laws governing these need to be reviewed.

The plant genetic resources are getting depleted at an alarming rate. It is estimated that there are more than 2,70,000 plant species in existence and about 34,000, i.e., 1 in 8, of these are endangered (IUCN, 1998). One major reason for the decline in plant genetic resources has been the explosion of human population and the resulting loss of habitat. One quarter of the plant species are estimated to be at risk of extinction within the next generation (Raven, 1987). Approximately one-fifth of the 20,000 native species in the United States are of concern, and possibly 800 of these may be lost in the next decade unless remedial measures are taken for their conservation (Centre for plant Conservation, 1995). In the context of India, 15-20% of the plant species are considered to be threatened. The plant genetic resources of the developing countries are more susceptible to threats due to destruction of habitat for inhabiting the ever-increasing human populations, unplanned developmental activities and ruthless exploitation of plants for commercial purposes. At times unmindful and excessive collection of plants by students of Botany may also endanger some rare plants. Another reason for the loss of valuable germplasms is the excessive introduction of species from one part of the world to another. The impact of the declining area under vegetation has resulted in a serious ecological imbalance *viz*. soil erosion and desertification, and dwindling of the forest wealth, wildlife and plant germplasm resources. Ecosystems contribute to climate regulation, maintenance of hydrological cycles and nitrification of soils. In addition, recreation, science and education also figure among the vast array of social, ethical, spiritual, cultural and economic goods and services provided by biodiversity that are recognized as fundamental for human livelihood and aspirations.

Unfortunately, in recent years, extinction has been the destiny of a great num-

ber of plant species including several unique and irreplaceable varieties, while many await a similar fate. Extinction of plant species means loss of opportunity to discover more useful forms. Genetic erosion, the reduction of diversity within the species, means losing the variation required for improvement of plants. Plant genetic resources have limits in their rates of growth, reproduction and adaptation. An additional loss of plant genetic resources on account of the destructive activities of man will jeopardise the welfare of future generations. The current rates of extinction demand immediate concerted efforts because, in the face of accelerating losses, our greatest enemy is time. Further, biological resources are likely to be the basis of all future welfare and security of nations.

Biotechnology in Conservation of Plant Genetic Resources

The role of biotechnology is to be seen as complementary to conventional conservation methods. Conservation of plant genetic resources could be accomplished both by *in situ* and *ex situ* methods. *In situ* conservation involves protection of genetic resources in the natural environment through the protection of the environment itself. It is an ideal and dynamic approach that allows plants to interact and co-evolve with other components of the ecosystem including insects, animals and microbes. However, this conservation method poses some problems. *In situ* conservation is costly to maintain and is highly susceptible to natural calamities like forest fires, extreme weather conditions, and damage by diseases and animals. On the other hand, Botanical Gardens have played an important role in *ex situ* conservation programmes particularly in acclimatization, rehabilitation, multiplication and judicious exploitation. Presently, there are about 1500 Botanical Gardens and Arboreta throughout the world and many countries are establishing new Botanical Gardens. In India, there are several age-old 'Sacred Forests', many of which are preserved to this day by traditional religious sanctions.

The tools of biotechnology are being increasingly applied now for conservation of plant genetic resources. This is especially true for the species with reproductive problems and/or extremely reduced populations. Recently, some efforts have been made to conserve DNA from plants and even from dried/fossil specimens of extinct taxa. The conserved DNA is expected to have many uses: molecular phylogenetics and systematics of extant and extinct taxa, production of previously characterized secondary compounds in transgenic cell cultures, production of transgenic plants using genes from gene families, *in vitro* expression and study of enzyme structure and function, and genomic probes for research laboratories (Adams, 1997). There are four main areas of biotechnology, which can directly assist plant conservation programmes: a) molecular marker technology, b) molecular diagnostics, c) *in vitro*

technologies, and d) cryopreservation.

Molecular Marker Technology and Diagnostics

The method for detecting genetic diversity in the past was based on Mendelian analysis, biometrical approaches and biochemical markers such as protein and isozyme profiles. Now the molecular marker techniques enable the assessment of plant diversity at the genomic level and plays major role in assisting plant conservation programmes (Ayad *et al*, 1997: Karp *et al*, 1997). The study of population structure and gene distribution patterns within the ecosystems can help support *in situ* conservation programmes. Contrasting examples of techniques include the assessment of restriction fragment length polymorphism (RFLPs) used for the detection of specific marker genes and polymerase-chain-reaction (PCR)-based technologies in conjunction with randomly amplified polymorphic DNA (RAPD) analysis. Molecular markers are of two broad types, high information content for a limited number of putative loci [e.g., microsatellites-simple sequence repeats (SSRs) and DNA sequence analysis (SA)] and low information content for a large number of putative loci [e.g., randomly amplified polymorphic DNA (RAPDs), and amplified frag)ment length polymorphism (AFLPs)]. The microsatellite sequences are short tandem repeats of mono- to tetra-nucleotide repeats which are assumed to be randomly distributed throughout the nuclear, chloroplastic and mitochondrial genome and are detected using specifically designed PCR primers (Jarne and Lagode, 1996). The DNA sequence analysis provides information of the nucleotide variation directly and aided with automated sequencing and high-powered computors is now being extensively used. The RAPD analysis utilizes single, arbitrary decamer DNA oligonucleotide primers to amplify regions of the genome usimg the PCR (Williams *et al*, 1993). The AFLP analysis involves the selective amplification of an arbitrary subset of restriction fragments generated by a lingle or double restriction enzyme digestion of DNA (Vos *et al*, 1995).

The identification of biodiversity based on marker surveys would not be complete unless the basis of variation is understood. The areas of data analysis and the understanding of molecular diversity in relation to the quantitative variation need further attention. The correlation between the molecular variation and quantitative variation has not been given enough attention. This study will be very useful in biodiversity assessment and conservation (Harris, 1999). The information generated by molecular markers must be linked with phenotypic expression of the quantitative characters. The direct practical applications of marker technologies are in the area of germplasm acquisition, plant diversity assessment, stability assessments of conserved plant germplasm and gene bank management (Bensen,

1999). The molecular tools help to identify particular populations or individuals with desirable traits, similarity between genotypes and available variations between the taxa, population or individuals. This would enable the researcher to decide the high priority genotype for conservation DNA inventories provide full details of the repositories and help in avoiding duplication of accessions and also in planning future missions for collection of missing germplasms. One of the risks associated with the collection of the plant germ plasm, especially from wild resources, is the inadvertant introduction of disease or pests along with the plant or seeds. Conservation of clonal plant germ plasm *in vitro* should assure that plants are protected from becoming infected with pathogens including viruses. It must also be emphasized that *in vitro* raised plants are disease free. All this can be ensured using immunological and molecular DNA methods, which is also an essential pre-requisite for transfer and international exchange of the disease-free germ plasm (Martin, 1998).

In vitro Propagation and Re-establishment

The advances in plant tissue culture have made significant contributions in the areas of cell biology, physiology, biochemistry and molecular biology (Bhojwani and Razdan, 1996; Raghavan, 1997). The more practical applications of the techniques are clonal multiplication, development of somaclonal variants, production of secondary metabolites, and improvement of plants through somatic hybridization and genetic manipulation. Plant tissue culture also holds great potential for conservation of germplasm (Prance, 1997; Feijoo and Iglesias, 1998; Lynch, 1999) by the use of micropropagation and embryo rescue. *In vitro* propagation methods led to re-establishment of endangered plant species in their natural habitat, for example the endemic Indian pitcher plant (*Nepenthes khasiana*) (Tandon and Rathore, 1994), some orchids (Seeni and Sabu, 1997), the lady's slipper orchid *Cypripediun calceolus* L (Ramsay and Stewart, 1998) in particular. The conservation of germplasm using *in vitro* methods is no exception to the inherent problems of specific culture requirements of different species, maintenance of morphological characters and genetic fidelity of the stored germplasm (Harding, 1999).

Micropropagation and re-establishment of plants involve the following basic steps -Stage 0: the preparative stage, involving germ plasm selection, Stage 1: the establishment stage, involving the production of axenic, viable cultures, Stage 2: the multiplication stage, during which the number of propagules is increased, Stage 3: the plantlet production, involving the development of germplasm of sufficient size and quality for transfer to *in vivo* conditions, and Stage 4: establishment under *in vivo* conditions, involving the acclimatization of plantlets to glasshouse con-

ditions (Lynch, 1999).

The collection of the germplasm is the first step for *in vitro* conservation. The seasonal effects significantly influence the initiation of cultures in many instances. In order to make germplasm collection from both managed and un-managed habitats, legal issues concerning the ownership, sovereignty and intellectual property rights also need to be considered (Guarino *et al*, 1995). The correct identification and collection of all possible diversity is essential without endangering the natural population. For the plant materials that are difficult to transport to the laboratory for the fear of loss of viability, inoculation of explants on the medium is carried out in the field with adequate facilities built in a mobile tissue-culture laboratory. The facilities of a mobile laboratory are quite simple and limited to the bare essential requirements. This approach has been successfully applied to a number of species, for example, coconut, cotton and cocoa (Withers, 1995; Ashmore, 1997). Compared to the conventional multiplication of plants, the micropropagation procedure has the advantage of rapid mass propagation of disease-free plants in a limited space. A large number of plants have been micropropagated using different explant sources, media and culture conditions. However, no generalization can be made with regard to the cultural practices used. The re-establishment of a large number of micropropagated plantlets into their natural habitat is of prime concern, as some endangered plants may not reproduce well by traditional methods. Several reviews on the use of *in vitro* propagation of endangered plants have appeared (Wochok, 1981; Fay, 1992, 1994; Fay and Gratton, 1992; Pence, 1999).

One of the pioneering programmes to use *in vitro* propagation methods for rare and endangered plants was started at the Micropropagation Unit of the Royal Botanic Gardens, Kew in 1974. Realizing the importance of conservation of Indian flora, the Ministry of Environment and Forests, Government of India initiated a major All India Co-ordinated Project on Conservation of Plants in 1985. 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. The National Bureau of Plant Genetic Resources (NBPGR) at New Delhi has been developed as a national facility for collection, evaluation, conservation and exchange of germplasm of a wide range of plant species. The National Facility for Plant Tissue Culture Repository was established in 1986 with funding from the Department of Biotechnology, Government of India and now has 926 *in-vitro* conserved plant accessions (Anon, 1999). Other important centres engaged in *in-vitro* conservation of plants are Indian Institution of Spices Research, Calicut; Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow; National

Table 1. Conservation of endangered Indian plant species using biotechnological methods

Family	Species	Status[1]	Propagation methods[2]	References
Acanthaceae	*Adhatoda beddomei*	Over-coll, med, few seeds, slow prop	Shoot tips	Sudha and Seeni, 1994
Amaryllidaceae	*Curculigo orchioides*	End, med, consv	Rhizome	Augustine and D'Souza,, 1997
Apocynaceae	*Rauwolfia micrantha*	Poor germ, poor rooting, med,	Shoot tips, nodes	Sudha and Seeni, 1996
	R. serpentina	Over-call, med, poor seed viability	Nodes, microprop	Sharma and Chandel,
	Wrightia tomentosa	Over-call, tree	Nodes	Purohit *et al.*, 1994a
Aquifoliaceae	*Ilex khasiana*	End,	Nodes, young leaf, microprop	Tandon *et al.*, 1990
Aristolochiaceae	*Aristolochia indica*	Over-coil, med	Shoot tips and nodes, leaf, adv sh	Manjula *et al.*, 1997
Asclepiadaceae	*Holostemma annulare*	Rare, med	Shoot tips, nodes	Sudha *et al.*, 1998
Asteraceae	*Saussurea lappa*	Rare, med	Shoot tips, microprop	Arora and Bhojwani, 1989
Combretaceae	*Anogeissus rotundifolia*	Rare, end	*In vitro* germs. microprop	Singh and Shekhawat, 1997
Dioscoreaceae	*Trichopus zeylanicus*	End, med	*In vitro* germ, microprop	Krishnan *et al.*, 1995
Fabaceae	*Pterocarpus marsupium*	Rare	*In vitro* germ, microprop	Das and Chatterjee, 1993
Gentianaceae	*Gentiana kurroo*	Over-coil, med, rare	Shoot tips, nodes callus	Sharma *et al.*, 1993
Lamiaceae	*Coleus forskohlii*	Over-coil, med	Nodes	Sharma *et al.*, 1991
Liliaceae	*Allium tuberosum*	Over-collection	Basal plate-adv.sh	Radhamani and Chandel, 1992
	Chlorophytum borivilianum	Over-coil, med	Shoot bases, microprop	Purohit *et al.*, 1994a
	C. borivilianum	Over-coil, med	Somatic embryo	Jain *et al.*, 1997
Lythraceae	*Woodfordia fruticosa*	Rare, med	Shoot tips, nodes	Krishna and Seeni, 1994
Nepenthaceae	*Nepenthes khasiana*	Rare, end	Shoot tips, nodes, microprop and re-establishment in nature	Tandon *et al.*, 1990; Rathore *et al.*, 1991; Tandon and Rathore, 1994
	N. khasiana	Rare, end	Shoot tips, nodes, microprop	Seeni, 1990; Latha and Seeni,1994
Nymohaeaceae				
	Nymphaea tetragona	Rare, end	Rhizome, embryos	Tandon *et al.*,1990

Family	Species	Status[1]	Propagation methods[2]	References
Orchidaceae				
	Dendrobium lindley	Rare	Microprop, *in vitro* germ	Kaur and Sarma,
	D. wardianum	Rare	Shoot tips, nodes microprop	Sharma and Tandon,
	Renanthera inschootiana	Rare	Leaf base, adv bud	Seeni and Latha, 199
Orchidaceae	*Vanda coerulea*	Over-coli, end	Leaf base, adv sh	Senni, 1990
	Vanila walkeriae	Prop for preserv	Nodes	Agrawal *et al.*,1992
Papaveraceae	*Meconopsis paniculata*	Poor seed germ & survival, hab loss	Callus-adv sh	Sulaiman, 1994
	M. simplicifolia	Hab loss, seedling mortality	Seedling explants- callus-adv sh	Sulaiman and Bab 1993
Podophylaceae	*Podophyllum hexandrum*	Over-coil, med	Zygotic embryo, callus -somatic embryo	Arumugam ar Bhojwani, 1990
Polygonaceae	*Rheum emodi*	Over-coil, med	Shoot tips	Lal and Ahuja, 1993
Ranunculaceae	*Aconitum heterophyllum*	Over-coil, med	Sh tips-microprop leaf, petiole-callus-somatic embryo	Giri *et al.*, 1993
	Coptis teeta	Rare, med	Hypocot, petiole, rhizome, microprop	Tandon and Rathor 1992
	Delphinium malabarcium	Low seed set, seed dormancy	Inflorese nodes	Agrawal *et al.*, 1991
Rutaceae	*Citrus assamensis* *C. indica* *C. latipes*	Rare	Sh tips, microprop	Baruah *et al.*, 1996
Scorphulariaceae	*Picrorhiza kurroa*	Rare, med	Shoot tips, nodes microprop	Lal *et al.*, 1988; Upadhyay *et al.*, 1989
Scorphulariaceae	*Limnophila indica*	Conserv	Root tips	Rao and Mohan Ram,1981
Sterculiaceae	*Steculia urens*	Over-coil	*In vitro* germ, microprop	Purohit and Dave, 1996
Valerianaceae	*Nardostachys jatamansi*	Over-coil, med	Petiole-callus-root adv sh	Mathur, 1992
	Valeriana wallichii	Rare, med	Sh tips, nodes, microprop	Mathur *et al.*, 1988

1. Abbreviations: hab, habitat; med, medicinal value; over-coil, over-collected; preserv, preservation; prop, propagation; rare endangered/threatened, consv, conservation; germ, germination.
2. Procedures abbreviations: adv, adventitious; hypocot, hypocotyl; imm, immature; microprop, micropropagation; sh shoot(s); germ, germination; inflorese, inflorescence.

Botanical Research Institute, Lucknow; National Chemical Laboratory (NCL), Pune; Institute of Himalayan Bioresource Technology, Palampur; G.B. Pant Institute of Himalayan Environment and Development (GBPIHED), Almora; Tropical Botanical Garden And Research Institute (TBGRI), Trivandrum; M.S. Swaminathan Research Foundation, Madras; Regional Plant Resources Centre (RPRC), Bhubaneshwar; Indian Council of Forestry Research and Education; and Departments of Botany at Delhi University, Delhi; North-Eastern Hill University, Shillong; Panjab University, Chandigarh; M.L.S. University, Udaipur; J. N. Vyas University, Jodhpur and many others. The Department of Biotechnology, Government of India has given tremendous impetus to conservation of plants using biotechnological approaches by establishing two micropropagation Technology Parks, 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 & Calcutta; 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. *In vitro* propagation techniques have been developed for many rare and endangered plants, of which some examples from the work in India are shown in table 1.

The micropropagation of endangered species is at times hampered due to limited availability of the material for raising cultures. The use of micropropagation protocols of the related non-endangered plants is resorted to in such cases (McComb, 1985; Campos and Pais, 1996). Other limitations in working with the endangered plant species include the lack of sufficient funds as compared to economically important plants, expensive field trips for collection of the material from remote and forested areas, and special permits for collection which are difficult to obtain and often time consuming.

The *in-vitro* seed germination has been extensively used for multiplication of rare orchid species, while many laboratories have taken up micropropagation of endangered cacti, succulents, insectivorous plants, lilies and several other groups of plants. Seeds are preferred for multiplication of rare and endangered species, as this would ensure genetic diversity. In certain cases, explants from the *in vitro* raised seedlings are used to initiate cultures for micropropagation. It is difficult to use explants from the field grown endangered plants for establishing cultures in many instances. Special pre-treatment of explants, media formulation and culture conditions need to be devised.

Short- to Medium-Term Storage

By reducing the growth rate the cultures can be stored for short to medium

term. This method is aimed at delaying the period of subculture without any detrimental effect to the plant tissue. This has been accomplished by reducing the temperature at which the cultures are grown. However, the responses of different cultures vary with respect to the lowering of temperature. The cold tolerant species such as strawberry and Prunus species can be stored at DOC to 4°C (Wilkins *et al*, 1998, Reed, 1992) but Musa plantlets cannot be stored below 15°C (Banerjee and Delanghe, 1985).

The slow growth of cultures can also be successfully obtained by incorporating osmotically active compounds such as mannitol (Staritsky and Zandvoort, 1985), reduction of growth regulators (Dussert *et al*, 1994) and strength of the nutrients (Malaurie *et al*, 1993) and by the use of a growth retardant (Jarret and Gawel, 1991). The mineral oil overlay of cultures has been tried to lower the oxygen levels. This enables a short-term conservation of germplasm (Constabel and Shyluk, 1994). Several plant species have been conserved *in vitro* at NBPGR, New Delhi by culturing them at room temperature (25°C) for varying periods between 6-24 months depending upon the species. The elegant procedure adopted does not involve the use of growth retardants. The cultures were shown to have 3-4 months of fast growth, then they entered into a stationary phase and were sustained on limited nutrients available to them for the next 6-8 months (Mandai, 1999). The slow-growth storage of cultures has certain drawbacks, for instance the management of large *in vitro* collections and possibility of development of somaclonal variations in cultures. However, Cassava stored under slow growth conditions for 10 years was found to be genetically stable (Angel *et al*, 1996).

Cryopreservation

There is a growing need for cryopreservation of plant genetic resources, which provides stable long-term storage in liquid nitrogen (LN) at -196°C. Cryopreservation can be applied to a wide range of materials, such as vegetatively propagated plants, recalcitrant seeds and even orthodox seeds (Harding *et al*,. 1997, Bensen and Lynch, 1998, Bensen et a/; 1998). Recently, increasing efforts have been made to cryopreserve endangered species (Pence, 1999) and tropical rain forest trees. Protocols for cryopreservation of a number of plant species are available now utilizing the methods of desiccation, slow pre-freezing and vitrification. During desiccation the free water is removed by transferring to the air. In slow pre-freezing, the free water inside the cells is transferred to extracellular ice during the slow freezing process (extracellular freezing). In vitrification extensive plasmolysis occurs and the free water is removed by subjecting the plant specimen to high osmotic solutions (Ishikawa, 1994).

A) Pre-treatments :

Pre-treatments are usually applied to germ plasm before cryopreservation. These include pre-culture of tissues: a) at lower temperatures to induce freezing tolerance, b) in the medium containing higher osmoticum to reduce tissue water and induce desiccation tolerance, and/or c) in media containing 'anti-stress' agents such as proline, abscisic acid or trehalose (Reed, 1996).

The application of simple dehydration pre-treatments in combination with sucrose and alginate bead encapsulation is an effective method for many species (Bensen, 1999).

B) Cryopreservation of recalcitrant seed species :

Several review articles have appeared in recent years which provide details on plants species whose embryos and/or embryonic axes have been successfully cryopreserved (Kartha and Engelmann, 1994; Pence, 1995; Engelmann *et al*, 1995; Engelmann 1997a and b). A perusal of these publications reveals that only a limited number of truly recalcitrant seeds have been cryopreserved compared to a large number of orthodox seeds which are routinely freeze preserved. The desiccation procedure is chiefly employed for freezing embryos and embryonic axes. The embryos or embryonic axes from recalcitrant seeds are desiccation intolerant. In certain cases, they are too large and not suitable for cryopreservation. However, in the case of tea, mahogany and neem, the seeds are relatively small and tolerant to desiccation and cryopreserved. They show low survival and problems with regard to regeneration. The successful examples of regeneration from cryopreserved material include: *Howea, Veitchia* and coconut (Chin *et al*, 1998; Assy-Bah and Engelmann, 1992). The area of cryopreservation of recalcitrant seeds needs more attention, as there is a large number of species with recalcitrant or suspectedly recalcitrant seeds from amongst the wild species. Further, in cases where seed storage conditions are known, regeneration from embryos and embryonic axes protocols, including inoculation *in vitro*, germination and growth of plantlets, propagation and acclimatization is often non-existent or not fully operational (Engelman, personal communication). This is particularly true for recalcitrant seeds of tree species in the tropics. The recalcitrant seeds do not exhibit any arrest in their development in contrast to orthodox seeds. Therefore, selection of the right developmental stage of the seed and modification of the regeneration medium may lead to an enhanced recovery rate of embryos and embryonic axes of recalcitrant seeds. Further, controlled desiccation, rapid desiccation and pre-growth of embryos on media containing cryoprotectant substances may also enhance the survivability. In cases where cryopreservation of embryos or embryonic axes have failed, the use of shoot

apices from the germinating embryos, adventitious buds or somatic embryos induced from the embryonic tissue may be attempted (Pence, 1995; Berjak *et al*, 1996). Attempts have been made at NBPGR, New Delhi to conserve recalcitrant seeds of mango, coconut, jackfruit, litchi, sapota and walnut. Successful cryopreservation using desiccation of embryonic axes followed by rapid freezing was achieved in tea, jackfruit, trifoliate orange and almond (Mandai, 1999). The crucial experiments for understanding the mechanism underlying seed recalcitrance and control of desiccation sensitivity will lead to improved cryopreservation of recalcitrant seed species the world over.

C) Traditional cryopreservation techniques :

The classical cryopreservation procedure involves slow cooling up to a defined pre-freezing temperature, followed by a rapid plunge into LN. The cells and external medium show super cooling during the slow cooling process and eventually ice formation occurs in the medium (Mazur, 1984). The cell membrane prevents the ice formation within the cell at this stage. As the temperature is further decreased an increase in ice formation in the extracellular medium occurs causing the concentration of the intracellular solutes. Under optimum conditions most or all of the intracellular freezable water leaves the cell. This reduces or avoids detrimental intracellular ice formation upon plunge of samples in LN. Glycerol, dimethyl sulphoxide (DMSO), sucrose etc. are routinely used as cryoprotectants. They exert their effect through colligative action and are considered stabilizing 'solvents' for the solute components of the frozen cells by preventing the damaging effects of cell dehydration and volume change. The cryoprotectants are able to penetrate the cell and as such, they are equally distributed in the extra -and intracellular compartments. During extracellular ice formation in the presence of cryoprotectants, water is lost by cell compartments that prevents the toxic concentration of the cell's solutes through dehydration. In addition, the damaging cell volume changes are prevented and the high cryoprotectant content of the cell would depress the freezing point to a very low sub-zero temperature at which damage is negligible or tolerated (Bensen, 1999). Thawing of frozen samples must be as rapid as possible to avoid the phenomenon of re-crystallization in which the ice melts and reforms at a thermodynamically favorable, larger and more damaging crystal size. The slow pre-freezing techniques are complex as they require the use of sophisticated and expensive programmable freezers and use of large amounts of LN. In some cases, the pre-freezing can be done using a laboratory freezer held at -30°C (Kartha and Engelmann, 1994; Tandon and Ishikawa, unpublished results).

The traditional cryopreservation methods have been successfully applied to un-

differentiated culture systems such as cell suspensions and calli. In the case of differentiated structures, these techniques can be used for freezing apices of cold-tolerant species (Reed and Chang, 1997). However, there are examples of successful use of these methods for tropical species such as cassava (Escobar *et al*, 1997).

D) New cryopreservation methods :

Vitrification has now become one of the main methods of cryoprotecting the plant germplasm for cryopreservation. In this method, cell dehydration is performed prior to freezing by exposure of the sample to concentrated cryoprotective media (usually comprising glycerol, DMSO, ethylene glycol and sucrose) and/or air desiccation. This is following by rapid cooling of the sample by plunging in LN. Vitrification is the process by which water undergoes a phase transition from a liquid to an amorphous 'glassy' state, which is not crystalline in structure (Sakai *et al*, 1990). This becomes possible when the solute concentration of the plant sample is so high that ice nucleation is prevented, thus the ice crystal formation and growth are inhibited by the highly viscous cell milieu.

Vitrification-based procedures offer several advantages in comparison to the traditional freezing techniques (Steponkus *et al*, 1992). These methods could be applied to complex organs (shoot tips, embryos) which possess a variety of cell types, each having unique requirement under conditions of freeze-induced dehydration (Engelmann, personal communication). The vitrification methods are very simple and do not require the use of controlled freezers. With adequate care these can be used for cryopreservation of samples collected directly from plants under field conditions.

The crucial step in vitrification is desiccation and not the freezing as in the case of traditional cryopreservation procedures. In cases where desiccation of samples below the critical level is possible no further drop in survival is generally observed after cryopreservation (Engelmann, 1997b). A number of different vitrification-based techniques are being followed. These are i) encapsulation-dehydration, ii) actual vitrification, iii) encapsulation-vitrification, iv) desiccation, v) pre-growth, vi) pre-growth desiccation, and vii) droplet freezing.

The encapsulation dehydration procedure involves encapsulation of explants in alginate beads, pre-growth in liquid medium (containing higher osmoticum using sucrose) for 1-7 days, further desiccation with air in a laminar flow table or over silica gel to a water content around 20% and then plunging into LN. Upon thawing the survival rate is high and regeneration mostly without intervening callus is possible. This procedure has been successfully applied to apices of numerous spe-

cies from temperate and tropical regions and also to cell suspensions and somatic embryos (Bachiri *et al*, 1995; Tessereau *et al*, 1994; Engelmann, 1997b). Vitrification has been used for cryopreservation of apices, cell suspensions and somatic embryos of different species (Sakai, 1995, 1997; Ishikawa *et al*, 1996). It involves the treatment of samples with cryoprotectant solutions, dehydration with highly viscous vitrification solution, rapid freezing, thawing, removal of cryoprotectants through washings with highly concentrated sucrose solutions, and recovery. Encapsulation-vitrification is based on the encapsulation of the material in alginate beads and their vitrification. Successful examples using this method are apices of carnation (Tannoury *et al*, 1991), 'wasabi' and Armoracia (Sakai, 1997). The desiccation method involves simple dehydration of samples and then direct plunge into LN. This procedure has been successful in cryopreservation of zygotic embryos or embryonic axes from a number of recalcitrant and intermediate seeds (Engelmann, 1997a). Dehydration is achieved using air current in a laminar flow table or by using sterile compressed air or silica gel. 'Flash drying' refers to the ultra-rapid dehydration using high pressure of sterile compressed air. This method facilitates the freezing of samples at a relatively higher water content (10-20% fresh weight basis). In pre-growth, the plant materials can be first grown in the medium containing cryoprotectants and then freezing them rapidly using LN. This procedure is successful for Musa apices (Paris, 1995). For pre-growth desiccation the explants are cultured in media containing cryoprotectants followed by dehydration in a laminar flow table or with silica gel and finally freezing the samples rapidly. This method has been used in asparagus stem segments, oil palm somatic embryos and coconut zygotic embryos (Uragami *et al*, 1990, Assy-Bah and Engelmann, 1992, Dumet *et al*, 1993). Droplet freezing has been used for a limited number of plant materials. Potato apices are pretreated with liquid cryoprotectant solution and frozen rapidly by plunge into LN (Schiafer-Menuhr, 1996).

Challenges for the 21 st Century

From the above description it is evidently clear that conservation, sustainable utilization and management of plant genetic resources will be the key to the survival and economic well being of human kind in the 21 st century. The rich plant genetic resources built over millions of years are under threat and there is an urgent need to conserve them for future generations. Documentation of plant genetic resources is essential using molecular markers such as RFLP, RAPD etc. Computer aided storage and retrieval systems of plant genetic resources will form an important tool for developing technology packages for conservation and ensuring exchange of information. An important aspect of this programme will be to prepare databases and provide information on patents. Adequate safe guards have

to be made against bio-piracy. In this context, patenting cells should be created in different parts of the country.

With the availability of FAa's world information and early warning system on plant genetic resource, its further erosion can be checked. In agriculture, diversity is threatened by the replacement of traditional land races by high yielding crop varieties and moving over to cash crops. It has been suggested that Agriculture Universities in India should act as 'Genetic Enhancement Centres' and provide scientific and technical services to farming community. These centres should also get a direct feed back from the farmers about their problems. There is a need to conserve much wider spectrum of germplasm from which to draw in times of need to broaden the genetic base of our productive system. Significant research efforts are required to understand biodiversity, genes and ecosytems as also the role of species diversity in communities. The northeastern region of India harbours about 50% of the flora and has been neglected in the past with regard to biodiversity conservation. It will be prudent to establish one major Botanical Garden (with tissue culture facilities) and two gene banks in the region and allocate sufficient funds for R&D activities in the area of plant genetic resource conservation and development of bioresources for the socio-economic upliftment of the local people.

Biotechnology will become an integral part of all aspects of germplasm acquisition, characterization, inventorization, conservation, exchange and genetic resource management. However, biotechnological techniques must not replace the traditional *ex situ* and in *situ* conservation methods, but rather provide complementary and enabling means of plant genetic resource management. Future prospects and needs must target certain key areas including: the development of appropriate structures for cryopreserved gene banks, the use of *in vitro* methods for the safe transfer of disease-free germplasm, and the application of genetic marker technologies for rationalizing germplasm procurement and gene banking. Unlike many biotechnological 'applications', conservation biotechnology programme must be considered with a long-term perspective.

Biodiversity has assumed enormous commercial importance. The use of medicinal and aromatic plants for cure of diseases with scientific understanding of active principles will gain importance. Bioprospecting of compounds, genes and species will help meet the needs of industry in future.

The Universities in the country should be encouraged to work on location specific problems related to biodiversity and development and conservation of biological resources. The subject fields of Conservation, Biotechnology and Biodiversity have become an integral part of curricula of colleges/universities in many coun-

tries. A compulsory course on conservation of biodiversity must be introduced at the undergraduate level and regular training courses for scientist and researchers be conducted in this area.

The state governments should be asked to give high priority to biotechnology based conservation of plant genetic resources in their plan, especially through the S&T councils. A close linkage between the universities-research institutions-industry must be established for concerted efforts in conservation of plant genetic resources and its management. The funding for R&D in the area of plant genetic resource conservation must be enhanced, particularly for the rare and endangered germplasm, an area that is often neglected.

The 'Information Technology revolution', aimed at distance learning and electronic networking specially designed for and targeted at plant conservation programmes in India, will promote the understanding of our rich germplasm and expedite international conservation activities. With new interpretations of Intellectual Property Rights issues the exchange of germ plasm will be possible with permission and suitable financial reimbursement would flow to the people who own the rich plant genetic resources in our country.

Biotechnology will contribute immensely towards conserving the vast plant genetic resources of the Indian subcontinent for posterity and underpin our ability to continue to meet new challenges. India with her vast plant genetic resources can be a big bargaining power among Nations in the 21st century. Accomplishment of this objective will also require continued public and scientific interest and political support.

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