Chapter 13
In Vitro Conservation Protocols for Some Threatened Medicinal-Plant

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13.1 Introduction

In the frame of global efforts to halt the loss of biodiversity by 2010, the Convention on Biological Diversity (CBD 1992; Glowka et al. 1994), and then the Global and European Strategies for Plant Conservation (GSPC, ESPC, available at www.plantaeuropa.org) have prioritized the *in situ* conservation of rare and threatened plant species and their back up by *ex situ* conservation in botanic gardens (Sharrock and Jones 2009). Recently the need to conserve plant diversity *ex situ* has been given added urgency as the impacts of climate change start to become obvious and concerns are raised about our ability to effectively conserve species *in situ* in the long run (Sharrock and Jones 2009). The 2008 IUCN Red List shows that the number of threatened plant species is increasing gradually. The number of threatened plants is 8,457, out of which 247 plants are found at different biodiversity hotspots in India. Many of them serve as sources of food, fuel, fibre, timber, medicine, etc. and function as integral parts of local agricultural production systems. The resurgence of public interest in plant-based medicine coupled with rapid expansion of pharmaceutical industries necessitate an increased demand of medicinal plants, leading to over-exploitation that threatened the survival of many medicinal plants.

Further, the degree of threat to natural populations of medicinal plants has increased because more than 90 % of the plant raw material for herbal industries in India is drawn from natural habitats. Not surprisingly, wild plant species used for medicinal purposes are receiving ever-increasing attention from the scientific community and commercial enterprises. At the same time, these species continue to support indigenous and local communities that have relied on them for centuries in their traditional medicines. But a number of factors now threaten wild medicinal

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plants – habitat destruction, over-harvesting and big business. In India, hundreds of medicinal plants like *Pterocarpus santalinus*, *Commiphora wightii*, *Taxus wallichiana*, *Picrorhiza kurrooa*, *Salvadora persica* and *Dioscorea deltoidea*, are at the risk of extinction due to over-collection to supply domestic and foreign medicinal markets, threatening the discovery of future cures for diseases. The special significance of medicinal plants in conservation stems from the major cultural, livelihood or economic roles that they play in many people's lives. Various sets of recommendations have been compiled relating to the conservation of medicinal plants, such as those associated with international conferences at Chiang Mai, Thailand, in 1988 and Bangalore, India, in 1998 (www.frlht-india.org) (Akerele et al. 1991; Bodeker 2002). Probably, the single most important 'role' for medicinal plants in biological and ecological conservation stems from the foundations that they can provide for the involvement of people in conservation of natural habitats (Schopp-Guth and Fremuth 2001). It is estimated that 70–80 % of people worldwide rely chiefly on traditional, largely herbal, medicine to meet their primary healthcare needs (Farnsworth and Soejarto 1991). The global demand for herbal medicine is not only large, but growing (Srivastava 2000). The market for Ayurvedic medicines is estimated to be expanding at 20 % annually in India (Subrat 2002), while the quantity of medicinal plants obtained from just one province of China (Yunnan) has grown by ten times in the last 10 years (Pei 2002). Factors contributing to the growth in demand for traditional medicine include the increasing human population and the frequently inadequate provision of Western (allopathic) medicine in developing countries. Natural and anthropogenic disturbances can have dramatic consequences for population growth, particularly for small populations of threatened plants.

Conservation of medicinal plants (MPs) is receiving increased attention in view of resurgence of interest in herbal medicines for health care all across the globe (Franz 1993; Gupta et al. 1998). The global market of herbal drugs has registered a steady increase in recent years (Martinez 1995, 1997; Olsen 1998), and now exceeds over US$ 20,000 million (Valiathan 1998). The world trade figures suggest that India ranks next to China in export (32,600 tonnes: US$ 46 million) of medicinal raw material annually (Lange 1997). All these figures indicate that MPs offer a great motivation for conservation (Marshall 1997) for all those concerned in human health care and economy. As a mega-diversity country, over 15,000 species of higher plants are so far recorded in India. Of these, nearly 50 % are reported to have medicinal value (All India Co-ordinated Research Project on Ethnobiology). In India, the rich plant diversity of the Himalaya is over 8,000 angiosperms, 44 gymnosperms, 600 pteridophytes, 1,737 bryophytes, 1,159 lichens etc. (Singh and Hajra 1996) and has been a source of medicine for millions of people in the country and elsewhere in the world. The Indian Himalaya region (IHR) supports over 1,748 (23.2 % of India) plant species angiosperms i.e., 1,685, gymnosperms – 12 and pteridophytes – 51 species of known medicinal value (Samant et al. 1998). The unique diversity of MPs in the region is manifested by the presence of a number of native (31 %), endemic (15.5 %) and threatened elements: 14 % of total Red Data plant species of IHR and 3.5 % of total MPs in different threat categories of CAMP (Samant et al. 1998). Medicinal plants of IHR are represented in varying life forms at various altitudes and habitats.

The economic potential of the MPs in some parts of IHR (Nautiyal et al. 1997) and their contribution in providing novel bio-molecules is recognised (Dhawan 1997). Further, the degree of threat to natural populations of MPs has increased because >90 % of medicinal plant raw material for herbal industries in India and for export is drawn from natural habitats (Tandon 1996; Gupta et al. 1998; Ved et al. 1998).

On the order of 40 % or more of the pharmaceuticals currently used in Western countries are already derived or at least partially derived from natural sources. Ayurveda, the indigenous system of Indian medicine, describes thousands of plant species in detail. With its varied climatic zones, India has a rich diversity of medicinal herbs. The forest harbour a large number of plant species, but deforestation has been responsible for the rapid loss of medicinal plant wealth, such that many valuable medicinal plants are under the threat of extinction. Pharmaceutical companies depend largely upon materials procured from naturally occurring stands that are being rapidly depleted. Application of traditional medicinal plants for human use has also been reported (Shimomura et al. 1997). Experimental approaches used for propagation of medicinal plants through tissue culture can be divided into three broad categories. The most common approach is to isolate organized meristems like shoot tips or axillary buds and induce them to grow into complete plants. This system of propagation is commonly referred to as micropropagation. In the second approach, adventitious shoots are initiated on leaf, root and stem segments or on callus derived from those organs. The third system of propagation involves induction of somatic embryogenesis in cell and callus cultures. This system is theoretically most efficient as large numbers of somatic embryos can be obtained once the whole process is standardised. Biotechnology involving modern tissue culture, cell biology and molecular biology offers the opportunity to develop new germplasms that are well adapted to changing demands.

13.2 Protocols of Some Threatened Plants

The following are some threatened plants, their importance, medicinal properties and their protocols of conservation through *in vitro* strategies:

13.2.1 Podophyllum hexandrum Royle

Podophyllum hexandrum Royle (May apple) belongs to the family Berberridaceae is a threatened perrenial herb and a source of highly valued podophyllotoxin. It is the rhizome of several *Podophyllum spp*. that has been found to be the source of podophyllotoxin, the active ingredient used as a starting compound for the chemical synthesis of etoposide (VP-16- 213) and teniposide (VM-26), effective agents in the treatment of lung cancer, a variety of leukemias and other solid tumours (Van Uden et al. 1989). The Indian species, *P. hexandrum* Royle contains three times more phyllotoxin than the American species *P. peltatum* (Fay and Ziegler 1985).

The rhizomes are being indiscriminately collected in large quantities from the wild to meet the ever increasing demand for the crude drug. As a result of this and a lack of organized cultivation, *P. hexandrum* has been reported as a threatened species from the Himalayan region (Gupta and Sethi 1983). The major problem for the cultivation of this plant is its long juvenile phase and poor fruit setting ability. Moreover, its seeds take long period to germinate (Handa et al. 1989). One of the most appropriate actions for safeguarding over exploited species is to improve propagation techniques and to encourage cultivation. Several studies have been conducted to propagate and conserve *P. hexandrum* by using tissue culture techniques like Arumugam and Bhojwani (1990). The reliable protocol for micropropagation of *P. hexandrum* is described below.

13.2.1.1 Protocol by Nadeem et al. (2000)

This protocol deals with the successful propagation of *P. hexandrum* using both conventional and *in vitro* techniques.

1. Vegetative multiplication was improved when rhizome segments were treated with α-napthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) before planting where more than doubling in rooting percentage was observed with 100 μM IBA.
2. Another experiment was carried out to improve the rate of seed germination where pre treatment of seeds with sodium hypochlorite resulted in five-fold increase, while 250 μM gibberellic acid (GA_3) and a combination of GA_3 and 6-benzyladenine (BA 250 μM each) enhanced germination by nearly two-fold increase.
3. The most reliable experiment of this protocol is the *in vitro* study of *P. hexandrum* and is as follows.
4. The embryos were excised from disinfected sterilized seeds of *P. hexandrum* and transferred onto MS (Murashige and Skoog 1962) medium containing 3.0 % sucrose (w/v) and 0.8 % agar (w/v) fortified with different plant growth regulators (PGR's) of varying concentrations.
5. Excised embryos germinated within 7 days of inoculation on basal medium or on medium supplemented with BA (0.5–4.0 μM).
6. Embryo expansion occurred rapidly when the medium was supplemented with 0.5 or 1.0 μM BA and the lowest concentration of BA resulted in maximum expansion.
7. Multiple shoot formation took place when the excised embryos were placed on MS medium supplemented with 1.0–4.0 μM IAA and 1.0 μM BA.
8. Highest shoot multiplication was observed on MS medium containing both IAA and BA (1.0 μM each) with a maximum of 5.0 shoots/embryo.
9. After 4–5 weeks, the base of the cotyledonary leaf of embryos swelled to give rise to multiple shoot formation.

10. Rooting of excised microshoots was carried out when the medium was supplemented with IAA.
11. Rooted plantlets were then hardened and transferred to polybags after 45 days and kept under a polyhouse.
12. Callus induced from the basal end of the embryo in most combinations gave rise to somatic embryogenesis when subcultured on a medium supplemented with 5.0 μM NAA and 0.5 μM BA.
13. For synthetic seed production the somatic embryos teased from embrygenic callus were embedded in 3.0 % Na-alginate solution and dropped into 5.0 μM $CaCl_2$ solution for hardening. The alginate beads were washed with sterilized distilled water.

13.2.2 Nothapodytes foetida (Wight) Sleumer

Nothapodytes foetida (Wight) an indigeneous small evergreen tree, belongs to the family Icacinaceae and the extract from this tree is used in the making of antileukaemia and antitumoural compound camptothecin. Clonal propagation of plant germplasm through tissue culture for rapid production of plants is an important prerequisite for in vitro conservation (Lynch 1999). Many of the indigenous species of India have not been investigated regarding their amenability to micropropagation (Mandal 1999). The most recent medicinal plant to come under serious threat from international traders is *Nothapodytes foetida*, a small tree found in rain forests of South India and Sri Lanka (Hoareau and DaSilva 1999). Govindachari and Vishwanathan (1972) reported the isolation of quinoline alkaloids camptothecin and 9-methoxy camptothecin from bark, stem, root, and leaf of the plant. A novel camptothecin derivative was isolated from the wood of this tree (Aiyama et al. 1998). A new naturally occurring alkaloid camptothecin and nothapodytines together with 17 known compounds were isolated and characterized from the stem of *N. foetida*. Several *in vitro* studies have been conducted to propagate and conserve *Nothapodytes foetida* like Thengane et al. (2001), Tejavathi et al. (2012). The most reliable protocol for this plant species is as follows.

13.2.2.1 Protocol by Ravishankar Rai (2002)

1. Sterilized seeds of *N. foetida* were inoculated on MS basal medium devoid of any plant growth regulators (PGR's).
2. After 4 weeks of germination hypocotyl segments were aseptically divided and cultured on MS shoot proliferation medium supplemented with different PGR's.
3. Among the different cytokinins tested viz. BA (Benzylaminopurine), KN (Kinetin), TDZ (Thidiazuron), 2.2 μM TDZ proved optimum with a maximum production of 18.3 shoots/explant.

4. Rooting was found to be optimum on 1/4 MS + IAA 5.7 μM + IBA 2.4 μM with a rooting percentage of 87.5 % and a mean root length of 3.2 cm.
5. After 4 weeks of successful acclimatization plantlets were transferred to large pots containing soil:compost (1:1) and kept in the greenhouse.

13.2.3 Allium wallichii Kunth

Allium wallichii Kunth (Alliaceae) is an erect herb of 30–40 cm height with slightly thickened and clustered bulbs Wawrosch et al. (2001). It is a perennial plant with a restricted distribution in higher altitudes ranging from 2,000–4,000 m above the sea level in Nepal (Malla 1976). The bulbous rhizomes are used for the treatment of coughs and colds and against altitude sickness. The young leaves are cooked as a vegetable and dried ones are used as a spice (Manandhar 1980; Dobremez 1982). *A. wallichii* is a common ingredient in stomach tonics. The bulb is also boiled, fried with ghee (clarified butter) and used for the treatment of cholera and diarrhea (Coburn 1984). In the Ayurvedic medicinal system, the indications are tuberculosis, nerve defects, blood circulatory defects, and long life and rejuvenation (Bajracharya 1979). Due to over exploitation of this plant species from its wild habitats it has achieved the status of threatened and is likely to be endangered in near future if the scenario remains the same. It has been noted that micropropagation would be of advantage in the process of the domestication of *A. wallichii* (Malla 1994). The application of tissue culture techniques has been described for various species of the genus Allium, for example garlic (Novak et al. 1986), onion (Dunstan and Short 1977; Rauber and Grunewald 1988), chive (Rauber and Grunewald 1988) or *A. carinatum* (Havel and Novak 1985). So far very reports on *in vitro* propagation and conservation of this plant species are available. One of the most acceptable protocol of *A. wallichii* is as follows.

13.2.3.1 Protocol by Wawrosch et al. (2001)

1. After proper disinfection and sterilization seeds of *A. wallichii* were aseptically germinated on half strength MS medium and the seedlings raised were multiplied on full strength MS medium supplemented with 10 μM BA to produce secondary explants which in turn were used for the main experiment.
2. Either whole shoots or longitudinally split halves (LH) were inoculated on the full strength MS medium supplemented with 108 factorial combinations of the cytokinins BA (6-benzyladenine), KN (kinetin) and Z (zeatin) and the auxins IAA (indoleacetic acid), IBA (indolebutyric acid) and NAA (napthaleneacetic acid).
3. An average multiplication factor of 4.6 was obtained when LH explants were inoculated on MS medium with 20 μM BA and 5 μM IAA and the same rate of shoot formation was also found on media containing Z.

4. Rooting was found optimum on 10 μM IBA with a rooting percentage of 100 % and a mean number of 2.22 roots with a root length of 12.2 mm per microshoot on full strength MS medium.
5. Acclimatization of *A. wallichii* plantlets was successfully done and subsequently the plants were moved to the greenhouse where they exhibited vigorous growth.
6. In addition to test the performance of *in vitro* derived plants under the specific conditions in Nepal a set of 300 shoots were rooted on MS medium with 10 μM IBA using disposable plastic containers.

13.2.4 Limonium cavanillesii Erben

The genus *Limonium* Miller (*Plumbaginaceae*), formerly called *Statice,* includes mainly rosulate plants with showy inflorescences (Amo-Marco and Ibanez 1998). This genus is characterized by its high ornamental value as a cut flower for both fresh and dry-flower arrangements (Harazy et al. 1985; Martin and Perez 1995). *Limonium cavanillesii* is an endemic and threatened statice with only a few populations known (Aguilella et al. 1994), and restricted to a small area in the coastal zone of north-eastern Castellon Province (Valencia Community, Eastern Spain).

Tissue culture techniques have proved to be good and efficient methods for the conservation of threatened plant species, because many plantlets can be obtained from a minimum of original plant material and with low impact on wild populations (Harazy et al. 1985; Lledo et al. 1993, 1994, 1996; Lledo and Amo- marco 1993; Martin and Perez 1992, 1995). One of the most reliable protocol for this threatened plant species is as follows.

13.2.4.1 Protocol by Amo-Marco and Ibanez (1998)

1. Inflorescence segments, 40–50 mm long, were excised and after proper sterilization 20 mm long explants with one or two buds were cut from the inflorescence segments and placed in MS medium.
2. When the shoots developed from buds of immature inflorescence stems were 1 cm in height and had 4–6 leaves, they were excised and subcultured on establishment medium with 2 mg/l kinetin.
3. For further multiplication, rosettes with 4–6 leaves were used as single explant.
4. Six week old shoots formed in clusters on establishment medium supplemented with 2 mg/l kinetin were selected and cultured on different multiplication media in combination with BAP, kinetin or 2iP at different concentrations.
5. Among the different cytokinins tested kinetin 5 mg/l, 2iP 5 mg/l and BAP 0.1 mg/l were found to be optimum where a maximum of 10.2, 9.4 and 9.3 shoots per cluster were obtained respectively.

6. Rooting was found to be optimum on 0.1 mg/l IAA where a maximum of 5.7 roots were produced with a rooting percentage of 80 % after 28 days.
7. Rooted plants were successfully acclimatized and transferred to plastic pots and the plantlets grew vigorously after the transfer with a survival rate of 90 %.

13.2.5 Decalepis hamiltonii Wight & Arn.

Decalepis hamiltonii Wight & Arn., commonly called 'swallow root', belongs to Asclepiadaceae and is a monogeneric climbing shrub native of the Deccan peninsula and forest areas of Western Ghats of India (Giridhar et al. 2005). The roots of *Decalepis hamiltonii* are used as a flavoring principle, appetizer, blood purifier (Wealth of India 1990), and preservative (Phadke et al. 1994). Of late, the highly aromatic roots have been subjected to over-exploitation by destructive harvesting that has endangered the survival of this plant in its wild habitat. Moreover, the absence of any organized cultivation of this plant (M. Sanjappa, Botanical Survey of India, personal communication) calls for immediate conservation measures. George et al. (1998) described a method for the preparation of the active fraction from the root of *Decalepis hamiltonii* and its use as a bioinsecticide. Also, it was observed that the aromatic roots of *Decalepis hamiltonii* proved to be potent antimicrobial agents (George et al. 1999). Though Bais et al. (2000) reported micropropagation of Decalepis hamiltonii by using growth hormones such as 6-benzyladenine (BA), a-naphthaleneacetic acid (NAA),n and indole-3-acetic acid (IAA), there were certain drawbacks such as profuse callusing from the base of the explants, and fewer shoots formed from nodal explants with moderate response (i.e., 30–40 %). A rapid and efficient protocol for micropropagation of *Decalepis hamiltonii* by shoot multiplication and effective *in vitro* rooting is reported by Giridhar et al. (2005) and is as follows.

13.2.5.1 Protocol by Giridhar et al. (2005)

1. Shoot tips of *D. hamiltonii* were used as explants source collected from Gumbali forest ranges of Mysore district.
2. The shoot tip explants were washed with Tween-20 (50 %, v/v) for 5 min followed by thorough washing under running tap water for 15 min. Later, explants were surface-sterilized with 0.15 % (w/v) mercuric chloride for 3–5 min and later rinsed four or five times with sterile distilled water.
3. For all experiments, MS (Murashige and Skoog 1962) with 100 mg/l myoinositol was used. All culture media contained 2 % sucrose (w/v). The pH was adjusted to 5.8 ± 0.2 using 1 N HCl or 1 N NaOH before adding 0.8 % (w/v) of agar (Himedia, Mumbai) and was subsequently autoclaved under 105 kPa at a temperature at 1,218 ° C for 15 min.
4. The MS medium with various concentrations of cytokinins such as isopentenyladenine (2iP; 2.5–7.5 μM), kinetin (2.3–7.0 μM), thidiazuron (2.3–6.8 μM) and

zeatin (2.3–11.4 μM) individually, or with IAA (0–0.85 μM), were investigated to optimize hormonal requirement for multiple shoot induction from shoot tip explants.

5. Of the various treatments with MS medium, 2iP (4.92 μM) alone produced the maximum number of shoots per culture (6.5 ± 0.4) that were supported by shoot length of 7.5 ± 0.5 cm after 8 week of culturing. Kinetin (4.7 μM) or zeatin (9.1 μM) in combination with IAA (0.6 μM) produced a maximum of 5.4 ± 0.4 or 5 ± 0.4 shoots, respectively.
6. Elongated microshoots were excised and transferred to MS medium fortified with phenolic compounds and indole-3-butyric acid (IBA; 9.8 μM) for root induction.
7. A greater number of roots (5.6 ± 0.8) were produced along with maximum root length (4.5 ± 0.3 cm) on medium containing 1,024 M PG (phloroglucinol).
8. At both concentrations of PG, 100 % of the explants responded for rooting.
9. After successful acclimatization these micropropagated plants were hardened under greenhouse conditions with 80–90 % survival rate.

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