Chapter 7
In Vitro Multiplication and Conservation of Threatened Medicinal Plants of Western Ghats of South India

R. K. Radha

Abstract Propagation of medicinal plants today is a promising alternative and counterpoint to wild collection, enabling preservation of natural genetic variability and survival of rare, endemic and endangered species, and it also provides quality raw material for pharmaceutical industries. Biotechnological methods like in vitro propagation technique hold tremendous potential for the production of high-quality plant-based medicines, which is an effective tool to conserve plant genes and guarantee the survival of the desired genotype, emphasised to make use of small units (cells and tissues) without losing their mother plant, thereby taking the pressure off from the waning wild populations and deriving a large number of plants in a very short time. Micropropagation protocols have worked out for many plant species cultured in vitro to provide macro – and micro-mineral nutrients, vitamins, source of carbohydrates under appropriate environmental conditions (light intensity, photoperiod and temperature) and plant growth regulators required to obtain high regeneration rates. In addition to the in vitro regeneration, germplasm conservation, reinforcement of genetic diversity and eco-rehabilitation of the waning medicinal plant taxa, it is very important to conserve and augment the resource supply. This chapter offers a brief insight into the status of micropropagation and mass multiplication strategies of elite genotypes, zygotic embryo cryopreservation of medicinal tree species and exploitation and utilisation of this technology for the conservation and ecorestoration of threatened or over-exploited medicinal plants in the tropical and subtropical regions of the Western Ghats, India.

Keywords In vitro · Conservation · Threatened medicinal plants · Multiplication

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Abbreviations

Auxin	Plant growth regulator assembling IAA in physiological activity
Axenic	Aseptic
BA	Benzylaminopurine
Callus	Disorganised meristematic or tumour-like mass of plant cells
Cytokinin	Plant growth regulator stimulating cell division and resembling kinetin in physiological activity. Mainly N_6 substituted aminopurine compounds
Explant	Excised fragment of plant tissue or organ used to initiate a tissue culture
IAA	Indole-3- acetic acid
IBA	Indole-3-butyric acid
JNTBGRI	Jawaharlal Nehru Tropical Botanic Garden and Research Institute
Meristem	Apical meristem culture; explant consisting only of apical dome tissue distal to the youngest leaf primordium
MS	Murashige and Skoog (1962) medium
MSL	*Mean* sea level
PGRs	Plant growth regulators
RET	Rare, endangered and threatened (RET) plants
NAA	Naphthalene acetic acid
SH	Schenk and Hilderbraandt (1972) medium

7.1 Introduction

The World Health Organization has estimated that more than 80% of the world population in developing countries depends primarily on herbal medicine for basic health care (Vines 2004; Peter et al. 2005; Krishnan et al. 2011), which accelerates the growth of herbal medicines in developed countries also. Subsequent global preference towards herbal medicine has advanced the expansion of plant-based pharmaceutical industries. Approximately two-thirds of the different medicinal plant species in use are collected from the wild, and in India, only 10% of medicinal species used commercially are cultivated. There is a growing concern about diminishing populations, loss of genetic diversity, extinctions and habitat degradation. Overexploitation and/or destructive harvesting to meet such demands, in fact, threatens the survival of many rare species (Krishnan et al. 2011; Tasheva and Kosturkova 2010). Confronted by such unprecedented genetic erosion and disappearance of species and ecosystems, conservation of natural resources assumes paramount urgency. In this perspective, micropropagation/in vitro clonal propagation techniques using shoot tip and nodal segments are indispensable to achieve mass multiplication and conservation of an endangered or threatened medicinal plant species within short period and limited space.

The interest in in vitro mass propagation of medicinal plants has distinctly increased as the method involves only organised meristems, allowing the recovery of genetically stable and true-to-type progenies, which is a major boon over the conventional methods of propagation. The advantages of micropropagation in medicinal taxa described by many authors (Krishnan et al. 2011; Eric et al. 2011; Sarasan et al. 2011; Mathe et al. 2015) are as follows: (i) In general, clonally propagated plants will have identical phytochemical profile independent of regional or seasonal variations. (ii) In many species, in vitro derived plantlets produced higher amount of desired compound than the normal plants. (iii) Usually multiple shoot cultures show stability of growth and secondary metabolite production characteristic to mature plants. (iv) In vitro shoots are used in the large-scale production of secondary metabolites. (v) In vitro shoots are also used for the long-term conservation and exchange of plant genetic resources.

It is also recommended to clone sufficient number of propagules collected from one source population to copy maximum genetic diversity (McGlaughlin et al. 2002), ensuring a self-sustained population of endangered species with full genetic diversity which is essential to salvage them from extinction (Falk et al. 2001; Eric et al. 2011; Sarasan et al. 2011). This route is seldom preferred by conservationists of India, but Jawaharlal Nehru Tropical Botanic Garden and Research Institute is one of the pioneer institutions to experiment with biotechnology-mediated curation of the waning medicinal plant taxa, which are employed over several countries in traditional system of medicine and in modern pharmaceutical industry through micropropagation, cryopreservation and recovery of the same through reintroduction into selected forest segments of the Western Ghats, India, thereby conserving and augmenting the resource supply.

7.2 In Vitro Propagation of Medicinal Plants Through Organogenesis

The development of reliable in vitro protocols is of great importance for conservation of threatened species by virtue of producing uniform planting material for offsetting the presence on the natural populations especially for medicinal plants. Application of both embryo and tissue culture facilitates rescuing the target species from the brink of extinction and establishment of viable populations in nature, contributing to eventual removal of them from the Red list. In vitro propagation protocols have been established for several thousand plant species, and many authors have reported encouraging results of plant regeneration from shoot tip and axillary meristems in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana* and *Digitalis* spp., *Rehmannia glutinosa*, *Isoplexis canariensis* (Paek et al. 1995; Perez-Bermudez et al. 2002), *Oroxylum indicum* (Dalal and Rai 2004), *Ginkgo biloba* (Tommasi and Scaramuzzi 2004), *Curcuma longa* (Prathanturarug et al. 2003), *Dendrobium candidum* (Shiau et al. 2005), *Curcuma zedoaria* (Loc et al. 2005),

Murraya koeningii (Rout 2005b), *Euphorbia nivulia* (Martin et al. 2005), *Clitoria ternatea* (Rout 2005a), *Tylophora indica* (Faisal et al. 2007) *Decalepis arayalpathra* (Sudha et al. 2005), *Tinospora cordifolia* (Raghu et al. 2006; Gururaj et al. 2007), C*urculigo orchioides* (Bhavisha and Jasrai 2003; Francis et al. 2007), *Glycyrrrrhiza glabra* (Vadodaria et al. 2007), *Swertia chirata* (Balaraju et al. 2009), *Picrorhiza kurroa* (Sood and Chauhan 2009), *Momordica tuberosa* (Aileni et al. 2009), *Withania coagulans* (Jain et al. 2009), *Ceropegia spiralis* (Murthy et al. 2010), *Aloe vera* (Singh and Sood 2009), *Aristolochia indica* (Soniya and Sujitha 2006), *Aristolochia tagala* (Animesh et al. 2007), *Rauvolfia serpentina* (Baksha et al. 2007), *Asparagus racemosus* (Nishritha and Sanjay 2008), *Vitex negundo* (Noman et al. 2008), *Baliospermum montanum* (Sasikumar et al. 2009), *Utleria salicifolia* and *Hemidesmus indicus* (George et al. 2010) and *Rubia cordifolia* (Radha et al. 2011), *Echinops spinosissimus* (Pan et al. 2003), *Elettaria cardamomum* (Nadganda et al. 1983; Bajaj et al. 1993), *Eleutherococcus koreanum* (Park et al. 2005), *Garcinia indica* (Malik et al. 2005), *Gloriosa superba* (Arumugam and Gopinath 2012), *Gynura procumbens* (Chan et al. 2009), *Hoslundia opposita* (Prakash and Van Staden 2007), *Hypericum perforatum* (Danova et al. 2012; Savio et al. 2012), *Labisia pumila* (Hartinie and Jualang 2007), *Leptadenia reticulata* (Kalidass et al. 2008), *Mollugo nudicaulis* (Nagesh and Shanthamma 2011), *Ornithogalum ulophyllum* (Ozel et al. 2008), *Ocimum gratissimum* (Gopi et al. 2006), *Peganum harmala* (El-Tarras et al. 2012), *Phyllanthus urinaria* (Kalidass and Mohan 2009), *Picrorhiza kurroa* (Jan et al. 2010), etc.

Micropropagation using seedling shoot culture has also been reported in *Camptotheca acuminata* (Liu and Li 2001), *Helleborus niger* (Seyring 2002), *Ophiorrhiza mungo* (Jose and Satheeshkumar 2004), *Origanum sipylum* (Oluk and Ali 2009) *Quercus semecarpifolia* (Sushma et al. 2008) and *Psidium guajava* (Shah et al. 2008), etc.

A number of reviews have been published on micropropagation, in vitro production of secondary metabolites and on field cultivation of medicinal plants; however, they do not provide the pragmatic standing of the protocol and scale-up production of plants that demonstrates the pilot-scale cultivation or continuous survival in the field. During the last two decades, various medicinal plants in threatened category which currently has high demand in pharmacuetical sectors have been sucessfully propagated and re-established in JNTBGRI by means of media optimization with supplementation of plant growth regulators and sucessful field establishment. Different regeneration pathways such as somatic embryos, callus-mediated shoot regeneration, direct regeneration without callus phase or with different explant sources including axenic seedlings were critically analysed in different species (Table 7.1) like Rauvolfia serpentina*, Rauvolfia micrantha, Justicia gingiana, Celastrus paniculatus, Trichopus zeylanicus, Nothapodytes nimmoniana, Decalepis arayalpathra, Piper barberi, Piper trichostachyon, Utleria salicifolia, Aristalochia tagala, Holostemma ada–kodien, Anaphyllum wightii, Coleus forskohlii,* Kaempferia galanga*,* Helminthostachys zeylanica *and Baliospermum montanum,* etc., to get optimum shoot multiplication (Fig. 7.2a–h), in vitro rooting and successful field establishment (Krishnan et al. 2011). Scale-up production and pilot-scale

Table 7.1 In vitro propagation protocols standardised in medicinal plants of the Western Ghats, India

Species (family)	Biome	Explant	Shoot proliferation medium with PGRs	Rooting medium and percentage establishment (%)	Explant and medium for subculture/mass multiplication
[a]*Mahonia leschenaultii* (Berberidaceae)	Palani Hills, Kodaikanal	Shoot tip/ node	SH + 1BA + 0.02IAA	MS + 1 IBA (72)	Node MS + 0.5BA + 0.01IAA
[a]*Heracleum candolleanum* (Apiaceae)	Peerumedu (Kerala) and Palani Hills (Kodaikanal)	Shoot tip/ node	MS+ 1BA	MS + 1 IBA (77)	Shoot tip/node MS + 0.5BA
[a]*Acorus calamus* (Acoraceae)	Prakashapuram (Kodaikanal) and Munnar (Kerala)	Rhizome with axillary bud	MS + 1BA + 0.5NAA	MS + 1 IBA (90)	Axillary bud/rhizome bud MS + 0.5BA + 0.2NAA
Kaempferia galanga (Zyngiberaceae)	Kallar Reserve Forest, Trivandrum (Kerala)	Rhizome With axillary buds	MS + 1BA+ 0.1NAA	0.2 IBA (85)	Axillary bud/rhizome bud MS + 1BA + 0.1NAA
[a]*Rubia cordifolia* (Rubiaceae)	Karadipara, Munnar (Kerala)	Shoot tip/ node	MS + 1BA + 0.5IAA	1 IBA (84)	Node/shoot tip MS + 0.5BA
Coleus forskohlii (Lamiaceae)	Salem, Tamil Nadu	Shoot tip/ node	MS + 1BA	1 IAA (98)	Node MS + 0.5BA
Rauvolfia serpentina (Apocynaceae)	Kanyakumari, Nilgiri Hills (Tamil Nadu)	Shoot tip/ node	MS + 0.5BA + 0.1IAA	MS+ 1IBA (80)	Node/shoot tip MS + 0.5BA + 0.1 IAA
Rauvolfia micrantha (Apocynaceae)	Kanyakumari, Nilgiri Hills (Tamil Nadu)	Shoot tip/ node	MS + 1BA + 0.5INAA	MS+ 1IBA (90)	Node/shoot tip MS + 0.5BA + 0.1 IAA
Justicia gingiana (Acanthaceae)	Malapuram, Thiruvananthapuram (Kerala), Coimbatore, Kanyakumari (Tamil Nadu)	Shoot tip/ node	MS + 1BA + 0.2 IAA	MS + 0.5 IBA (95)	Node MS + 1BA + 0.2IAA

(continued)

Table 7.1 (continued)

Species (family)	Biome	Explant	Shoot proliferation medium with PGRs	Rooting medium and percentage establishment (%)	Explant and medium for subculture/mass multiplication
Curcuma longa (Zyngiberaceae)	All districts in Kerala	Rhizome with axillary bud	MS + 3BA	Rooting was spontaneous in all the treatments (99)	Shoot tip/node MS + 1BA
Helminthostachys zeylanica (Ophioglossaceae)	Kannur, Malappuram, Thiruvananthapuram (Kerala)	Rhizome bud	WPM + 1 BA	WPM + 1 BA (69)	Shoot bud WPM + 1 BA
[a]*Myristica malabarica* (Myristicaceae)	Kuzhathupuzhaand Sendurnai forests, Kerala	Shoot tip/ node	MS + 1BA + 0.2 NAA	MS + 0.5 IBA (75)	Node/MS +1BA
Curcuma aromatica (Zyngiberaceae)	Palakkad, Kasaragode, Wayanad, Thrissur, Pathanamthita, Kollam, Idukki, Thiruvananthapuram, Kozhikode (Kerala)	Rhizome with axillary bud	MS + 3BA	Rooting was spontaneous in all the treatments (99)	Shoot tip/node MS +1BA
Trichopus zeylanicus (Dioscoraceae)	Southern Western Ghats, Kollam, Thiruvananthapuram (Kerala)	Rhizome with axillary bud	MS + 2BA + 0.5NAA	MS + 0.5 IBA (80)	Node MS + 0.5BA
Celastrus paniculatus (Celastraceae)	Palakkad, Idukki, Malapuram, Kannur, Thrissur, Wayanad, Kozhikode (Kerala)	Shoot tip/ node	MS + 1BA	MS + 0.2 IBA (90)	Node MS + 0.5BA
Nothapodytes nimmoniana (Icacinaceae)	Idukki (Kerala)	Shoot tip/ node	MS + 1BA	MS + 0.2 IBA (90)	Node MS + 0.5BA
Decalepis arayalpathra (Periplocaceae)	Kallar reserve Forest, Bonacaud Forest (Kerala)	Shoot tip/ node	MS + 0.5BA	MS + 1IBA (80)	Node MS + 0.5BA
Piper barberi (Piperaceae)	Southern Western Ghats, Palakkad, Thiruvananthapuram, Idukki, Kollam, Thrissur, Wayanad	Shoot tip/ node	MS + 1BA	MS + 0.5BA (89)	Node MS + 0.5BA

Species (family)	Biome	Explant	Shoot proliferation medium with PGRs	Rooting medium and percentage establishment (%)	Explant and medium for subculture/mass multiplication
Piper trichostachyon (Piperaceae)	Palakkad, Idukki, Pathanamthitta, Kollam, Wayanad (Kerala)	Shoot tip/ node	MS + 1BA	MS + 0.5BA (85)	Node MS + 0.5BA
Utleria salicifolia (Periplocaceae)	Southern Western Ghats, Palakkad, Idukki (Kerala)	Shoot tip/ node	MS + 0.5BA	MS + 1IBA (80)	Node MS + 0.5BA
Baliospermum montanum (Euphorbiaceae)	All districts in Kerala, Coorg, Chikmagalur, Karnataka	Shoot tip/ node	MS + 0.5BA	MS + 1IBA (80)	Node MS + 0.5BA
Holostemma ada-kodien (Asclepidaceae)	All districts in Kerala	Shoot tip/ node	MS + 0.5BA	MS + 1IBA (90)	Shoot tip/node MS + 0.5BA

[a]Reintroduced in the natural forest segments of the tropical and subtropical regions of Southern Western Ghats, India

cultivation trials of mericlones regenerated from rhizome bud/axillary bud explants were explored (Krishnan et al. 2011) in rhizomatous plants like *Curcuma longa, Curcuma aromatica, Kaempferia galangal* and *Acorus calamus* (Fig. 7.3). Different species (*Mahonia leschenaultii, Heracleum candolleanum,* Acorus calamus*, Rubia cordifolia and Myristica malabarica*), which are employed over several countries in pharmaceutical industry, are also critically examined by the author for conservation through micropropagation and recovery of the same through reintroduction into selected forest segments of the Western Ghats, India.

Quick and large-scale production of clonal plants through in vitro regeneration of single node/shoot tip/ axillary bud explants, and its subsequent shoot proliferation obtained in *M. leschenaultii, H. candolleanum, A. calamus, R. cordifolia and M. malabarica* can be portrayed as the best example of how in vitro protocols increase the rate of multiplication over hundred-fold in comparison to the conventional methods. This eases the ways of obtaining explants for deliberate establishments of species into an area and or habitat where it has become extirpated (Tables 7.1 and 7.2).

Successful in vitro regeneration procedure in *Mahonia leschenaultii Nutt.*, an endemic small tree of the Western Ghats with excellent source of berberine having antitumour properties, was achieved through multiple axillary shoot formation in single node cultures. A synergistic combination of 1.0 mgl^{-1}BA and 0.02 mgl^{-1}IAAin Schenk and Hildebrandt (SH) induced the maximum number (5.9) of axillary shoot formation which were relatively high (75%) when the explant collected during May–June and fifth node from the top of the growing shoots were used. Repeated subculture of the nodes from shoot cultures at 5–6 week intervals in medium supplemented with reduced concentrations of the growth regulators (0.5mgl^{-1} BA, 0.01 mgl^{-1} IAA) through at least 10 passages enabled consistent production of 6–7 shoots (Fig. 7.1a) per node at 92% success rate without loss of vigour, growth and morphological abnormalities. Shoots of 3–6 cm were rooted in vitro in the presence of 1.0 mgl^{-1} IBA (Fig. 7.1b) and hardening in the mist house at 76–78%, and this rooted plants were established in a potting medium of river sand and top soil (1:1) under constant mist irrigation. The plants reared in the nursery for 5–8 weeks were successfully transferred into the natural forest segment of the institute's campus (MSL 200 m) revealed an establishment frequency of 78.75 after 18 months (Radha

Table 7.2 Medicinal plants of the Western Ghats micropropagated through direct shoot regeneration with experimental trials conducted for restoration/translocation in forest habitats

Species	Establishment in native/alien localities (%)	Observed period (months)
Acorus calamus	90/85	36
Heracleum candolleanum	85/90	48
Mahonia leschenaultii	80/75	24
Rubia cordifolia	90/85	36
Myristica malabarica	90/80	24

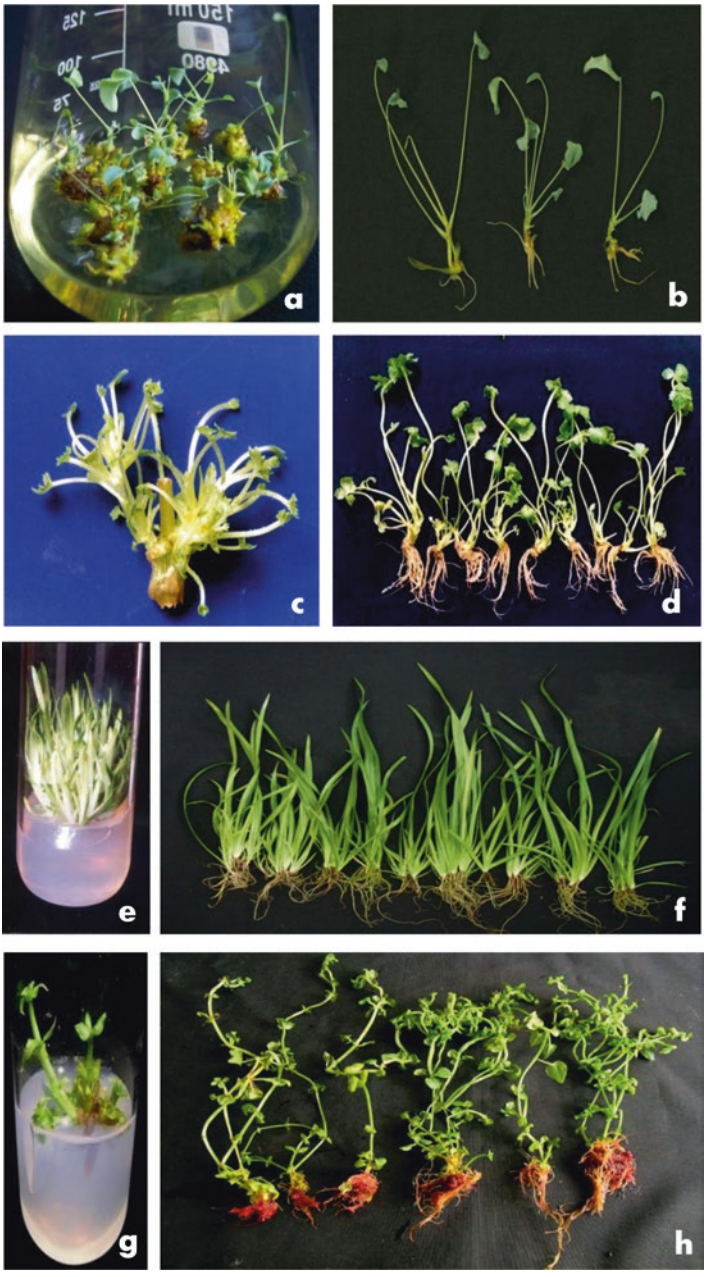

Fig. 7.1 In vitro shoot proliferation and rhizogenesis in threatened medicinal plants of the Western Ghats, India. (**a**) Shoot proliferation from the nodal explants (SH + 0.5 BA and 0.01 IAA) in *M. leschenaultii*. (**b**) Rooted meiclones of *M. leschenaultii* (SH medium + 1 IBA). (**c**) Shoot proliferation from the nodal explants (MS + 1 BA) in *H. candolleanum*. (**d**) Rooted mericlones of *H. candolleanum* (MS medium + 1 IBA), (**e**) Shoot proliferation from the nodal explants (MS + 1 BAP and 0.5 NAA) in *A. calamus*. (**f**) Rooted mericlones of *A. calamus* (MS medium + 1 IBA). (**g**) Shoot proliferation from the nodal explants (MS +1 BA and 0.2 IAA) in *R. cordifolia*. (**h**) Rooted mericlones of *R. cordifolia* (MS + 1 IAA)

et al. 2013). Conventional vegetative propagation of this small tree distributed along the margins in high-altitude evergreen forests between 1600 and 2400 m in the southern Western Ghats is slow while outright clearing of the natural stands due to increased human inhabitation and conversion into hill crop areas especially in the Palani Hills and Nilgiri Hills of Western Ghats posing danger to its survival. Perusal of the literature also revealed very little information on tissue culture of this species, though in vitro propagation of berberine-rich *Berberis thunbergii* (Karthu and Hakala 1991), high berberine-producing cells of *Coptis japonica* (Sato and Yamada 1984) and bioproduction of berberine in callus tissues of *Thalictrum minus* (Ikuta and Hokawa 1982) and cell cultures of *Coscinium fenestratum* (Nair et al. 1992) are reported. The ready availability of micropropagated systems as demonstrated in *M. leschenaultii* may spur economic cultivation of the species for future industrial raw material supply, if it is developed as an economic crop for the extraction of berberine.

High-frequency microcloning of *Heracleum candolleanum* (wight&Arn.) Gamble., an important medicinal plant endemic to India with limited geographical distribution recorded across the Western Ghats of Karnataka (Bababudan Hills of Chikmagalur), Kerala (Peermade) and Tamil Nadu (Palani Hills in an altitude range of 1500–2300 m) regions of southern India and considered vulnerable/global, was established through callus-free axillary meristem cultures on Murashige and Skoog (MS) medium supplemented with cytokinin alone (1.0 mgl^{-1} BA); a maximum of 9.8 shoots (Fig. 7.1c) were formed in the nodal explants. Shoots were multiplied by routine periodic subcultures through 6-week intervals and 1.0 mgl^{-1} IBA favoured the development of 4.24 roots within 5 weeks of culture (Radha 2011) and rooted plants of *H. candolleanum* preferred a mixture of river sand, soil and farmyard manure (1:1:1). Micropropagated plantlets transplanted into forest segments in the institute's campus (MSL 200 m) followed by their growth characteristics free of abnormalities confirm their utility in conservation through revegetation of the denuded forest segments in the Western Ghats.

In the process of efficient shoot proliferation from axillary bud explants of *Acorus calamus* L., (vulnerable, semi-aquatic perennial) a combined influence of BA and IAA (Fig. 7.1e), 13.9 resulted in the production of shoots after an incubation of 30 days. Each bud thus raised rooted profusely (~14 roots with 80%) in medium supplemented with 1.0 mgl^{-1} (Fig. 7.1f) of any of the said auxin type (IBA,IAA,NAA) to produce 13 plantlets. On recurrent subculture, fresh flush of shoots raised more than 15 plants after every 30 days from the mother culture, resulting in the stocking of approximately 115 plants (Fig. 7.3) at the end of the first subculture in contrast to the published results (Rani et al. 2000; Anu et al. 2001). Formation of aromatic rhizome was first noticed in the 10 months after field transfer and then onwards rhizome continued to grow under the soil in length and breadth simultaneously producing aerial leaves from the nodes. The repeated cultivation of rhizomes of shoots at 8–9 month intervals in specially prepared bed of soil and mud (5 × 5 m) favoured profusion of shoots and production of rhizome, the useful part of the plant containing essential oil; these processes would be better achieved through

bulk supplies of propagules for planting in diverse localities through biotechnology-mediated multiplication than by conventional means. This is despite the fact that agro technology for cultivation of *Acorus calamus*is already developed using rhizome cuttings. Demand for Sweet flag in the world market is growing in pharmaceutical industry as production of syrups, balms and medicated candies; it is also used in combination with Basil, Brahmi and other herbs as popular health supplement for memory booster, immunity enhancer and tonic, and its smell makes calamus essential oil valued in the perfume industry.

The highly traded medicinal plant *Rubia cordifolia* Linn. (Manjishtha/Indian Madder) contains substantial amounts of anthraquinone especially in the roots; plants distributed sparsely in the lower hills of Indian Himalayas in the North and Western Ghats in the south showed remarkably efficient in vitro shoot regeneration and rooting capacity, both of which are significantly influenced by the varying concentrations of the different plant growth regulators. The optimum number of shoots obtained was 5.9 and 5.2 per explant in 2 weeks on the medium supplemented with 1mgl^{-1}BA and 0.02 mgl^{-1} IAA in nodes (Fig. 7.1g) and split vertical halves of the node, respectively. Shoot multiplication was rapid and consistent for four subcultures with 0.5mgl^{-1}BA. The best root induction (98%) and survival was achieved on 1 mgl^{-1} IBA followed by 1 mgl^{-1} IAA (Fig. 7.1h). Micropropagated plants displayed normal phenotypes in ex situ conditions with 89% survival. These plantlets can be used to replenish declining populations in the wild, for the extraction of bioactive compounds and reducing pressure on wild stocks (Radha et al. 2011).

Nodal explants of germinated axenic seedlings of *Myristica malabarica* Lam., a threatened tree species, when introduced into half MS medium with 1.5 mgl^{-1} BA and 0.2 mgl^{-1} NAA with activated charcoal (1 gm) induced multiple shoot formation (Table 7.1). Sprouting of axillary buds on the lower nodes (mature nodes) of the seedlings was obtained with the addition of 5 gl^{-1} adenine sulphate. Supplementation of the medium with auxin was essential for rooting of adventitious shoots (1.0 mgl^{-1} IBA). More importantly, the investigations prove beyond doubt the efficacy of shoot regeneration from axillary bud explants of plants raised from the seedlings and zygotic embryos with cotyledons and successful field establishment (90%) (Figs. 7.2 and 7.3).

7.2.1 Conservation Through Micropropagation and Ecorestoration

The establishment of a plant species as a stable component of a plant community is widely regarded as the most desirable process of species conservation and will be achieved only through reintroduction of micropropagated plants into its native habitat. Many authors (Wochok 1981; Maunder 1992; Fay 1992, 1994; Frankel et al. 1995; Wyse and Sutherland 2000; Eric et al. 2011) have emphasised the importance of this critical requirement for rare plant conservation. Falk et al. (1996, 2001) stress

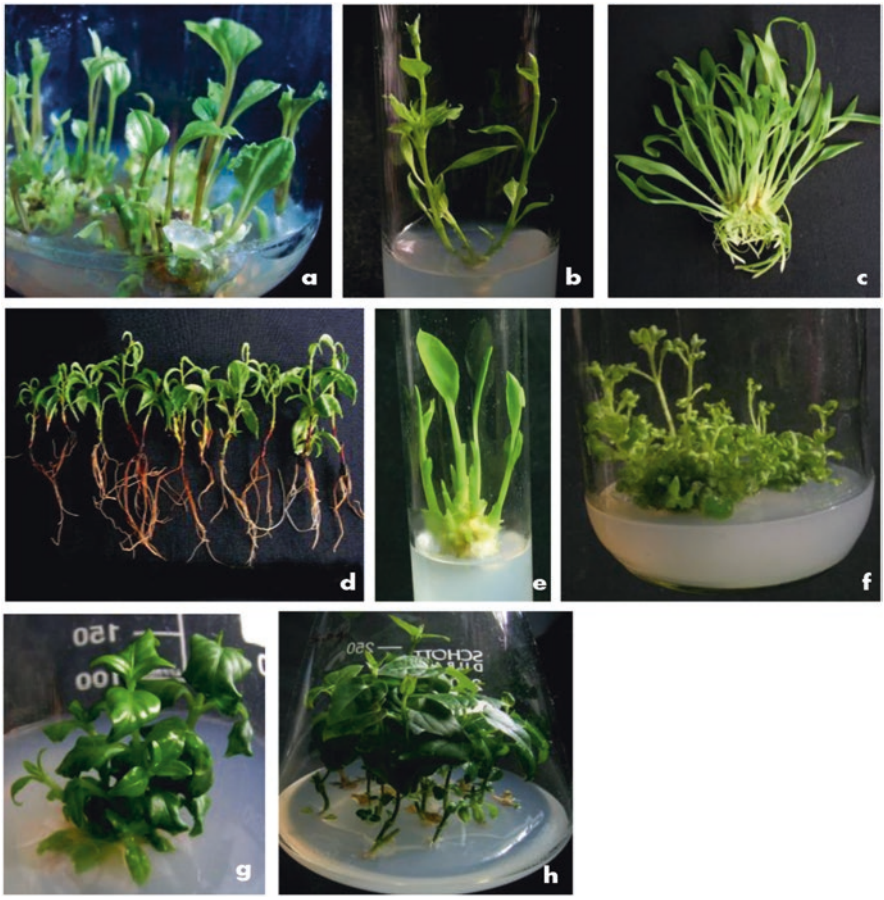

Fig. 7.2 In vitro shoot proliferation in threatened medicinal plants of the Western Ghats, India. (**a**) *Trychopus zeylanicus.* (**b**) *Decalepis arayalpathra.* (**c**) *Kaempferia galanga.* (**d**) *Celastrus paniculatus.* (**e**) *Curcuma longa.* (**f**) *Coleus forskohlii.* (**g**) *Rauvolfia serpentina.* (**h**) *Holostemma ada-kodien*

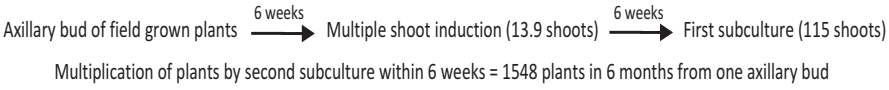

Fig. 7.3 Rate of multiplication of *A. calamus* by tissue culture

the importance of conservation strategies, involving in situ and ex situ preservation as well as reintroduction. Reintroduction/ecorestoration is the deliberate establishment of individuals of RET species into an area and/or habitat where it has become extirpated with the specific aim of establishing a viable self-sustaining population for

conservation purposes. In fact, the goal of reintroducing endangered species is to reverse decline in the distribution and abundance that have been caused directly or indirectly by human activities. The intention is to ascertain self-sustaining populations that retain the genetic diversity necessary to undergo evolutionary change (McGlaughlin et al. 2002). Many species reintroduced into its native habitats have been growing well and the technology has already been successfully demonstrated by many authors in *Paphiopedilum rothschildianum* (Grell et al. 1988), *Bletia urbana* (Rubulo et al. 1989), *Ipsea malabarica* (Gangaprasad et al. 1998; Martin 2003), *Calophyllum apetalum* (Lakshmi and Seeni 2003), *Blepharistemma membranifolia* (Lakshmi and Seeni 2001), *Decalepis arayalpathra* (Gangaprasad et al. 2005), *Vanda coerulea* (Seeni and Latha 2000), *Vanda spathulata* (Decruse et al. 2003), *Syzygium travancoricum* (Anand 2003), Bulgaria golden root (Tasheva and Kosturkova 2010), *Ceropegia fantastica* (Chandore et al. 2010) and *Rhododendron ponficum* (Almeida et al. 2005). As a part of our continued efforts to conserve rare, endangered and endemic plants of conservation value through in vitro propagation and reintroduction, experimental ecorestoration of mericlones of five medicinal plants, *Mahonia leschenaultia* (Palani Hills of Kodaikanal), *Heracleum candolleanum* (Palani Hills of Kodaikanal), *Acorus calamus* (Palani Hills of Kodaikanal), *Rubia cordifolia* (Karadipara, Munnar) *and Myristica malabarica* (Sendurnai forest ranges), was successfully attempted during 2000–2016. About 100–500 plants were reintroduced into their native (Table 7.1) or alien habitats (forest patches of institute campus) recorded 75–90% establishment after 1–2 years (Table 7.2). Plants reintroduced into forest segments of the Western Ghats with favourable microclimatic conditions performed better with high-percentage establishment and profuse growth as evidenced from formation in quick succession of new leaves in relation to that of the plants in the institute campus (Krishnan et al. 2011; Radha 2011; Radha et al. 2013). Periodical monitoring of the establishment of reintroduced plants after 5 years also showed promising response of growth, flowering and seed set. Overall, this study comprising the development of an in vitro propagation protocol, mass propagation and recovery of the plants through reintroduction into native and alien habitats together provides a comprehensive package for conservation and sustainable utilisation of all the experimental species. The establishment of viable populations in sites (forest patches of institute) other than their natural habitats (translocation) is also desirable as it facilitates the survival of the species in more than one ecologically conducive site.

7.2.2 Ex Situ Conservation Through Cryopreservation

Long-term ex situ storage of plant germplasm is of increasing importance, both for maintaining the genetic diversity of species with existing human use and for the preservation of species threatened with extinction in the wild; seed banks have long been used for this purpose. Most of the agricultural species are desiccation tolerant or orthodox seeds, which are often viable for many years, and their longevity can be

increased further by storing the seeds at a very low temperature (−196 °C), in liquid nitrogen (LN). In contrast, number of species have desiccation sensitive or recalcitrant seeds; tropical timber, fruit and plantation crops as well as species from several threatened habitats fall into this category (Berjack et al. 2011; Noor et al. 2011). Recalcitrant seeds are generally short lived, are often large with considerable quantities of fleshy endosperm and cannot be stored intact using traditional methods of drying. In vitro conservation offers alternative techniques for the long-term preservation of this plant germplasm, consisting of slow growth techniques and cryopreservation. While slow growth is for short- to medium-term storage, cryopreservation of zygotic embryos, shoot tip/meristems and pollen play a major role in the long-term conservation of tropical plants with recalcitrant seeds. Seeds of many species are too large to be frozen directly, so desiccation technique is mainly employed for freezing embryos and embryonic axes (Engelmann 2004) which has been confirmed for the investigations regarding cryopreservation of woody species like *Myristica malabarica, Nothapodytes nimmoniana, and Celastrus paniculatus* (Radha et al. 2006, 2010a, b) at JNTBGRI (Table 7.3). In *N. nimmoniana* and *C. paniculatus*, excised zygotic embryos subjected to simple desiccation under laminar air flow for 60 min reduced moisture content to 19.6 and 31.8, 60 to 66% of them regenerated into whole plants upon LN storage. Excised zygotic embryos of *M. malabarica*, *M dactyloides* and *M. undapine* subjected to desiccation for 120 min are also suitable for cryopreservation to get 56–65% whole plant regeneration, but the desiccation trials on *C. fenestratum* is not promising, as cryopreserved embryos recorded only 34% germination against 56% in desiccation control (60 min). Experiments suggest that isolated embryos of *C. apetalum* are tolerant to 2 hrs desiccation with 53% survival. However, successful cryostorage was achieved

Table 7.3 Zygotic embryo cryopreservation protocols standardised for medicinal trees of tropical and subtropical regions of Western Ghats, India

Species	Category of seeds	Desiccation time (min.)	Moisture content after desiccation (%)	Percentage survival after LN storage
Myristica malabarica	Recalcitrant	120	31.7	60
Myristica dactyloides	Recalcitrant	120	39.9	65
Myristica undapine	Recalcitrant	120	39.8	56
Nothapodytes nimmoniana	Intermediate	60	19.6	60
Coscinium fenestratum	Intermediate	60	37.3	34
Celastrus paniculatus	Recalcitrant	60	31.8	66
Calophyllum apetalum	Recalcitrant	120	20.6	53

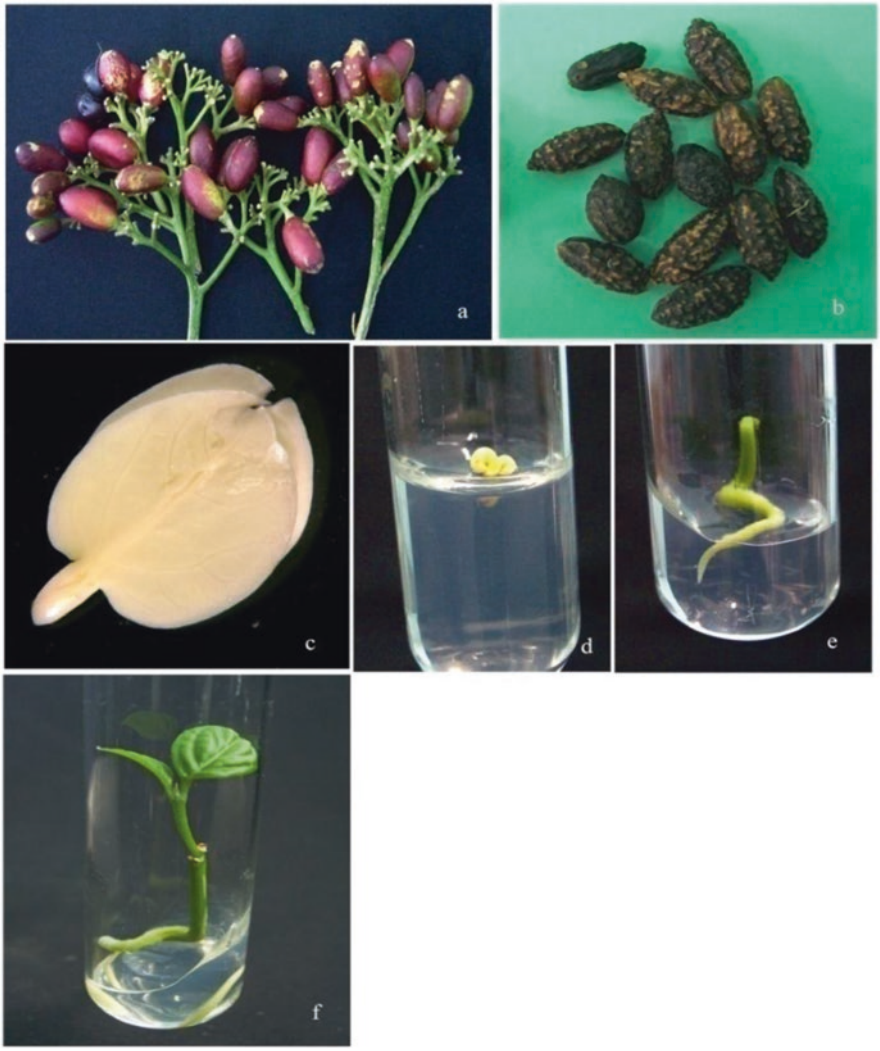

Fig. 7.4 Plant regeneration from cryopreserved embryos of *Nothapodytes nimmoniana.* (**a**) Fruits. (**b**) Seeds. (**c**) Embryo. (**d**) Cryopreserved embryo showing germination. (**e**) Cryopreserved embryo developed into seedling (30 days). (**f**) Cryopreserved embryo developed into seedling (60 days)

for most of the dehydration-tolerant seeds (Figs. 7.4, 7.5, and 7.6); this situation together with an ease to develop independent plants in vitro from embryonic axes may provide an effective technique for the long-term conservation of desiccation sensitive woody medicinal plants of the Western Ghats (Radha et al. 2010a, b; Krishnan et al. 2011).

Fig. 7.5 Plant regeneration from cryopreserved embryos of *Myristica malabarica.* (**a**, **b**) Fruit and seed from two accessions. (**c**) Embryo of two accessions. (**d**–**h**) Cryopreserved embryo showing germination and development. (**i**–**k**) Fruit, zygotic embryo, and germinating LN treated zygotic embryo of *Myristica dactyloides*

7.3 Conclusion

The efficiency of regimented micropropagation system, rehabilitation, understanding the phenomenon of seed recalcitrance and comprehensive cryopreservation practices are thus proved effective for the conservation and ecorestoration of threatened medicinal plants of the tropical and subtropical regions of Western Ghats, India. However, the reintroduction carried out in these published work of our laboratory is on an experimental scale. In order to realise ecological restoration, extended planting of plantlets in more than one locality is a mandate. Sufficient numbers of

Fig. 7.6 Plant regeneration from cryopreserved embryos of *Celastrus paniculatus*. (**a**) Fruits. (**b**) Ripened fruits. (c) Embryo. (**d**) Germination of LN treated embryo. (**e**) Cryopreserved embryo developed into seedling (30 days). (**f**) Cryopreserved embryo developed into seedling (60 days)

propagules are recommended to be cloned from one source population to mirror utmost genetic diversity, fortifying a self-sustained population of endangered species with the precise genetic diversity essential to extricate them from extinction. The exact number of plants that needs to be reintroduced varies with species and heterogeneity of the source population. In addition, the experiments with optimisation of scale-up production in different culture vessels including airlift bioreactors can support in the development of more effective propagation and storage technologies. In fact, most of the pharmaceutically important medicinal plants have not been micropropagated on large scale or reintroduced more than one region which is a major glitch in the current scenario. As the extinction pressures are increasing, it is important that priority species are identified for scaling up of shoot cultures and establishment of demonstration stage cultivation at pilot-scale level to conserve and commercialise production of therapeutic plants utilising all the premier biotechnological tools available.

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